Development of Multilocus PCR Assays for *Raffaelea lauricola*, Causal Agent of Laurel Wilt Disease

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Abstract

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Laurel wilt, caused by the fungus *Raffaelea lauricola*, is an exotic disease that affects members of the Lauraceae plant family in the southeastern United States. The disease is spreading rapidly in native forests and is now found in commercial avocado groves in south Florida, where an accurate diagnostic method would improve disease management. A polymerase chain reaction (PCR) method based on amplifying the ribosomal small-subunit DNA, with a detection limit of 0.0001 ng, was found to be suitable for some quantitative PCR applications; however, it was not taxon specific. Genomic sequencing of *R.*

lauricola was used to identify and develop primers to amplify two taxon-specific simple-sequence repeat (SSR) loci, which did not amplify from related taxa or host DNA. The new SSR loci PCR assay has a detection limit of 0.1 ng of *R. lauricola* DNA, is compatible with traditional and real-time PCR, was tested in four labs to confirm consistency, and reduces diagnostic time from 1 week to 1 day. Our work illustrates pitfalls to designing taxon-specific assays for new pathogens and that undescribed fungi can limit specificity.

The ambrosia beetle symbiont, *Raffaelea lauricola*, is a unique pathogen of trees that has created an unprecedented phenomenon in American forests, laurel wilt (10,15,19). Typically, ambrosia fungi are nonpathogenic or weakly pathogenic to their tree hosts (19,27) but *R. lauricola* has challenged this paradigm in dramatic fashion. *R. lauricola* is transmitted by the Asian redbay ambrosia beetle (*Xyleborus glabratus*), which is not known to cause damage in its native range (19,30). Less than 2 years after its appearance in an area, more than 92% of the redbays >7.6 cm in diameter and 100% of those greater than 10.2 cm in diameter were killed (10,12). As of August 2013, the disease was found in North and South Carolina and Georgia, across most of Florida (45 of the 67 counties), and in Mississippi and Alabama (39,31).

Laurel wilt affects members of the Lauraceae plant family, including redbay (*Persea borbonia*) (9,10), swamp bay (*P. borbonia* var. *pubescens*) (23), silkbay (*P. humilis*) (18), sassafras (*Sassafras albidum*) (34), and camphor (*Cinnamomum camphora*) (35). Three shrubs (northern spicebush [*Lindera benzoin*], pondberry [*L. melisifolia*; endangered], and pondspice [*Litsea aestivalis*; critically endangered]) are also susceptible (8,17,37). Substantial losses of a fruit crop, avocado (*P. americana*) (20,25,28,29), are developing in commercial groves in Florida (29). In Florida alone, avocado is worth in excess of \$60 million annually, and more valuable production is threatened in California and Latin America (7).

Management of laurel wilt is limited to injection of trees with systemic fungicides prior to infection (24) and the judicious use of sanitation, particularly in avocado orchards (36). It has been demonstrated that the vector and fungus do not survive in wood that has been chipped (36). Thus, early detection of symptomatic trees

and subsequent sanitation may slow the development of laurel wilt epidemics, particularly in areas devoid of significant densities of natural hosts. Monitoring based on trapping the vector is problematic because trapping efficiencies for *X. glabratus* are poor and the disease often develops before it is detected. In addition, several additional potential vector species have been identified (4,14).

Currently, diagnosis of laurel wilt requires recovery of *R. lauricola* on a semi-selective medium (29), followed by sequencing of the small subunit (SSU; 18S) or large subunit (LSU; 28S) of the ribosomal DNA (rDNA) or the demonstration of pathogenicity for examined isolates (*unpublished data*). No taxon-specific polymerase chain reaction (PCR) assay for the pathogen has yet been reported. The aim of this study was to develop reliable and accurate real-time and traditional PCR-based methods to identify *R. lauricola* and diagnose laurel wilt.

Materials and Methods

DNA extraction. The DNA extraction method for samples used in this study was adapted from Justesen et al. (21). Wood samples were ground using a mortar and pestle with liquid nitrogen. Fungal conidia or mycelia samples (10 to 50 mg) were lysed by adding 0.8 ml of extraction buffer and three cycles of freezing at -80°C for 5 min followed by incubating at 65°C until thawed. The ground wood samples were suspended in 0.8 ml of extraction buffer (D-sorbitol at 25 g liter $^{-1}$, N-lauroylsarcosine at 10 g liter $^{-1}$, hexadecyltrimethylammonium bromide at 8 g liter $^{-1}$, 0.8 M NaCl, 20 mM EDTA, polyvinylpyrrolidone at 10 g liter $^{-1}$, and 0.1 M Tris, pH 8) and 2 μl of RNase A (100 mg ml $^{-1}$) was added; the mixture was inverted five times and incubated at 65°C for 25 min. The mixture was cooled to room temperature and 0.8 ml of chloroform was added; the result was inverted 30 times and centrifuged for 5 min at 16,000 relative centrifugal force (RCF). The supernatant was transferred to new 2-ml tubes, 20 μl of proteinase K (20 mg μl^{-1}) was added, and the suspension was inverted four to six times and incubated for 20 min at 55°C . The tubes were cooled to room temperature and 0.8 ml of chloroform was added; the tubes were then inverted 30 times and centrifuged for 5 min at 16,000 RCF. The supernatant was transferred to new 2-ml tubes, one volume of ice-cold 100% isopropanol was added, and the tubes were inverted and centrifuged for 15 min at 16,000

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RCF. The DNA pellets were washed with 350 µl of 75% ethanol and centrifuged for 5 min at 16,000 RCF; ethanol was removed, and then the pellets were air dried and resuspended in 100 µl of Tris-EDTA buffer. PCR was conducted on all DNA extractions using the rDNA SSU primers NS1 and NS4 (40) to confirm that all DNA extractions contained amplifiable DNA before using. DNA from *R. lauricola* PL1390 culture was used to construct a serial dilution, which was used to determine the detection limit and as a positive control. DNA was obtained for *R. brunnea*, *R. albimanens*, *R. ambrosiae*, and *R. tritirachium* from the Centraalbureau voor Schimmelcultures and for *R. canadensis* C592, *R. monteyi* C2221, *R. albimanens* C2223, *R. brunnea* C2229, *R. subfusca* C2253, and *R. fusca* C2336 and C2349 from Dr. T. C. Harrington (Iowa State University).

SSU-based detection method. An alignment of multiple 18S SSU rDNA sequences of *R. lauricola* and closely related species, retrieved from GenBank, was evaluated for potential *R. lauricola* taxon-specific PCR primer sites. Three primers—PCR primers LWD1 and LWD3 that matched only *R. lauricola* but later produced a false positive and were discarded, and a second set of qPCR primers, LWD3 and LWD4, that matched *R. lauricola* and *R. brunnea*—were designed. PCR conditions for LWD3 and LWD4 were optimized by testing a range (50 to 60°C) of annealing temperatures. The primers were also tested against DNA from an *R. lauricola* serial dilution, both redbay and avocado laurel-wilt-diseased (naturally infected and artificially inoculated with isolate PL159, respectively) and healthy wood, and DNA from related fungi: *R. canadensis*, *R. monteyi*, *R. albimanens*, *R. brunnea*, *R. subfusca*, and *R. fusca*. The thermocycling protocol was 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 56°C for 15 s, and 68°C for 30 s, with a melting curve analysis performed at the end. SYBR green was used as the detection method. The PCR mixture included 11.25 µl of Eppendorf 2.5× Realmastermix/SYBR

solution, 10.75 µl of water, 1 µl of LWD3 10 µM primer, 1 µl of LWD4 10 µM primer, and 1 µl of DNA template. All PCRs were replicated in triplicate with the *R. lauricola* serial dilution and no-DNA negative control present on every run using an Eppendorf Mastercycler ep Realplex, and Realplex 2.0 software was used for all quantitative (q)PCR data analysis (Eppendorf Inc.).

Microsatellite-based detection method: sequencing and primer design. One-eighth of a Roche 454 GS FLX Titanium sequencer plate was used to generate sequences from *R. lauricola* isolate (PL716 gq996063) by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida, Gainesville. Primer_designer.pl (5; modified by M. Gitzendanner) was used to find simple-sequence repeats, also known as microsatellites, and design primers to amplify them. Design parameters specified that each repeat contain a minimum of eight repeat units and primers not be located within 25 bp of the repeat. The primers were then sorted by the number of repeats (large to small), repeat unit size (small to large), and product size (small to large). The 454 sequences were assembled using Geneious 5.4.6 de novo assembler using the high sensitivity option, with all other options left to default settings (3).

Microsatellite-based detection method: primer screening. Microsatellite primer pairs were first prescreened to limit the total number of PCRs. The prescreening consisted of testing the primer pairs with DNA from *R. lauricola* PL1390 to confirm that the correct amplicon was produced; and from its two closest relatives, *R. brunnea* (22) and *Raffaelea* sp. PL1004 (undescribed fungus that produced the false positive with the LWD1-LWD3 SSU-based method), to confirm that no amplicons were produced. Primer pairs that passed the prescreening were then tested against all of the isolates (Table 1). The primers were tested against DNA from *Raffaelea* spp. from the two clades closest to *R. lauricola* (*R. monteyi*, *R. sulphurea*, *R. brunnea*, *R. ellipticospora*, *R. ambrosiae*, *R. subfusca*, and *R. fusca*; 13) and included representatives (*R. subalba*, *R. tritirachium*, *R. albimanens*, and *R. arxii*) from more distantly related clades (13). DNA from healthy redbay and avocado was included in the screening process to insure that the primers would not produce a false positive from host material.

The selected *R. lauricola* taxon-specific primer pairs, chk and ifw (GenBank KF381410 and KF381411 respectively), were further tested against nine isolates of *R. lauricola*. This was done to confirm that the primers produced the correct amplicon for all isolates. Six isolates were from the United States; three contained polymorphisms (PL388, PL692, and PL735) and three were identical to all other isolates (PL159, PL570, and PL571) according to an amplified fragment length polymorphism study (M. Hughes, *personal communication*). Two isolates, CBS 129001 and CBS 129006, from Taiwan, and one isolate, CBS 129007, from Japan (16), were also tested. The amplicons from the two primer pairs for the nine isolates were sequenced using Sanger sequencing at the University of Florida's ICBR.

Microsatellite-based detection method: PCR conditions. The optimal annealing temperature for the primer prescreening was determined by testing a range of temperatures (60 to 68°C). A temperature of 65°C was chosen as optimal because it was the highest temperature that did not reduce amplification and the higher temperature would increase PCR stringency when compared with the lower temperatures (41). The thermocycle profile consisted of 95°C for 180 s, followed by 40 cycles of 95°C for 35 s, 65°C for 30 s, and 72°C for 60 s, with a melting curve analysis performed upon completion. All PCRs used in this experiment were replicated in triplicate with *R. lauricola* positive controls and non-template negative controls present for every PCR run using an Eppendorf Mastercycler ep Realplex (Eppendorf Inc.). All PCR products were then visualized on agarose gels. Four DNA polymerases were tested: Biolase, (Bioline USA Inc.), AmpliTaq, AmpliTaq Gold, and AmpliTaq Gold 360 (Life Technologies Corp.), following the manufacturers' instructions for each. AmpliTaq Gold was used for the primer screening because it produced the lowest overall cycle threshold (Ct) values.

Table 1. Isolates used to screen *Raffaelea lauricola* specific microsatellite primers

PL number	Other names	Species or notes
1008	...	<i>Paraphaeosphaeria</i> sp.
1009	CBS 271.70	<i>Raffaelea albimanens</i>
893	C2750	<i>R. amasae</i>
894	CBS 185.64	<i>R. ambrosiae</i>
896	C2372	<i>R. arxii</i>
1194	CBS 378.68	<i>R. brunnea</i>
890	C2345	<i>R. ellipticospora</i>
891	C2394	<i>R. fusca</i>
159	Avocado B	<i>R. lauricola</i> Duval County, FL
388	Pierce County	<i>R. lauricola</i> Pierce County, GA
570	MK1	<i>R. lauricola</i> Clay County, FL
571	MK2	<i>R. lauricola</i> Clay County, FL
692	...	<i>R. lauricola</i> Appling County, GA
716	MS2	<i>R. lauricola</i> Jackson County, MS
717	MS3	<i>R. lauricola</i> Jackson County, MS
735	Columbia County	<i>R. lauricola</i> Columbia County, FL
1467	CBS 129007	<i>R. lauricola</i> Japan
1468	CBS 129001	<i>R. lauricola</i> Taiwan
1469	CBS 129006	<i>R. lauricola</i> Taiwan
1007	...	<i>R. lauricola</i> from <i>Ambrosiodmus lecontei</i>
894	C2221	<i>R. monteyi</i>
1001	1073	<i>Raffaelea</i> sp.
1002	1080	<i>Raffaelea</i> sp.
1003	1081	<i>Raffaelea</i> sp.
1005	...	<i>Raffaelea</i> sp. from <i>Xyleborus affinis</i>
1000	3649	<i>Raffaelea</i> sp. from <i>X. ferrugineus</i>
996	3645	<i>Raffaelea</i> sp. from <i>X. glabratus</i>
999	3648	<i>Raffaelea</i> sp. from <i>X. glabratus</i>
1006	...	<i>Raffaelea</i> sp. from <i>Xyleborus</i> sp.
1004	RL5	<i>Raffaelea</i> sp. from dead <i>Persea americana</i>
892	C2401	<i>R. subalba</i>
889	C2335	<i>R. subfusca</i>
895	C593	<i>R. sulphurea</i>
896	CBS 726.69	<i>R. tritirachium</i>

PCR reactions for the primer screening consisted of 2.5 µl of GeneAmp 10× PCR Gold buffer (Life Technologies Corp.), 2.5 µl of 2 mM deoxynucleotide triphosphates (Bioline USA Inc.), 3 µl of 25 mM MgCl₂, 1 µl of 10 mM forward primer, 1 µl of 10 mM reverse primer, 0.4 µl of 10× SYBR Green I solution (Lonza Inc.), 0.125 µl of AmpliTaq Gold DNA polymerase (Life Technologies Corp.), 13.475 µl of H₂O, and 1 µl of DNA template. All of the primers used in this study were synthesized by Integrated DNA Technologies, Inc.

After the taxon-specific primers were chosen, a range of primer annealing temperatures (60 to 65°C) was tested. A fifth DNA polymerase (Immolase (Bioline USA Inc.) was also tested, which produced the same detection limit as AmpliTaq Gold, produced more amplicons, costs less, and was used in subsequent testing. The PCRs were the same as above, except that 1.5 µl of 50 mM MgCl₂ and 14.975 µl of H₂O were used. The thermocycle profile for Immolase DNA polymerase consisted of 95°C for 4 m, followed by 35 cycles of 95°C for 35 s, 65°C for 30 s, and 72°C for 60 s, with a melting curve analysis performed upon completion. The primer pairs chk and ifw (Table 2) were chosen for the diagnostic test because they did not produce amplicons in the presence of related taxa or host DNA and amplified well from *R. lauricola* DNA. The taxon-specific primers and the above methodology were tested in four independent laboratories (Carrie L. Harmon, Aaron J. Palmateer, Randy C. Ploetz, and Jason A. Smith) to confirm their reliability.

Results

SSU-based detection. The primers LWD1 and LWD3 (Table 2) produced an 832-bp amplicon and, when a 68°C primer annealing temperature was used, the primers did not produce amplicons in the presence of *R. amasae*, *R. arxii*, *R. ellipticospora*, *R. fusca*, *R. montetii*, *R. subalba*, *R. subfusca*, *R. sulphurea*, or healthy redbay and avocado DNA. However, when used to examine a suspect, dead avocado tree in August 2009, they resulted in a spurious diagnosis for *R. lauricola* or laurel wilt. A fungus from this tree, *Raffaelea* sp. PL1004, resembled *R. lauricola* phenotypically and produced a SSU sequence (GenBank KF026302) that was 99% (971/980) similar to *R. lauricola* (JF797172, e-value of 0.00). However, it was not pathogenic to avocado in two pathogenicity tests (R. C. Ploetz, unpublished) and is now considered to be a closely related but undescribed *Raffaelea* sp.

The qPCR primers LWD3 and LWD4 (Table 2) have a detection limit of 1×10^{-4} ng of *R. lauricola* DNA per qPCR. The standard curve constructed using the serial dilution (6.3 to 1×10^{-4} ng per qPCR) produced a slope of -4.193 , PCR efficiency of 0.73 , R^2 of 0.997 , and Ct standard deviation of 0.64 at the lowest limit. There was no amplification or primer dimer formation with the no-DNA template, healthy avocado, or redbay-wood negative controls. However, at least one replicate of each related *Raffaelea* sp. tested produced an amplicon with approximately the correct melting temperature. However, the amplification occurred late in the amplification process (mean Ct values > 30).

Microsatellite-based detection. During 454 sequencing, 80,237 reads were generated with an average length of 324 bp. Among the sequences, Primer_designer.pl found 3,103 microsatellites that contained fewer than eight repeat units, 3,117 microsatellites without suitable flanking primers, and 212 microsatellites with suitable primers. The sequences containing the chk and ifw loci were located in the 454 sequence assemblies to confirm that they were not

in the same contig, adding evidence that they were located in different parts of the genome. Both of the *R. lauricola* taxon-specific primer pairs had a detection limit of 0.1 ng of *R. lauricola* DNA per PCR and were able to quickly screen DNA from cultures but were not able to reliably detect *R. lauricola* from diseased wood samples (see Supplementary Figures 1 to 5 for all gel images and additional information). The primer pairs produced the correct amplicon for all isolates of *R. lauricola* that were tested. The six *R. lauricola* isolates from the southern United States and the three from Asia had identical DNA sequences for the ifw locus, and all except the CBS 129007 isolate from Japan, which had 14 additional TCT repeats, had the same chk sequences. All four labs and Carrillo et al. (4) were able to use the microsatellite-based detection method to screen suspect *R. lauricola* isolates, confirming the consistency of the methodology.

Discussion

Given the emergent nature of this pathogen and its potential threat to natural forests and avocado culture globally, surveillance programs have been initiated in several countries (Australia, Israel, Mexico and South Africa). The need for a practical diagnostic method that is consistently reliable in different labs is imperative. To date, diagnostic methodology has relied on sequencing of the LSU or SSU; however, the time and extra resources needed for this approach make it unrealistic for many diagnostic labs.

The false positive that was obtained with the SSU (LWD1 and LWD3 primers) and *Raffaelea* sp. (PL1004) illustrates the difficulty of designing taxon-specific PCR assays for new pathogens, as well as the dangers that are associated with the use of a single marker or locus for pathogen identification. These problems are especially acute when unknown, closely related taxa are present. To address these hurdles, microsatellite loci were used as the basis for a new method to detect *R. lauricola*. Microsatellites are ideal for such an objective because they have limited intertaxon transferability (2,6), vary in length (33), and have been used previously to detect pathogens (1). In addition, numerous methods have been developed to locate microsatellites and design primers for their amplification (26,38,42). The primers that were designed for the chk and ifw loci in the present study distinguished different isolates of *R. lauricola* from Asia and the United States from a wide array of closely related taxa. Their accuracy and successful use in four different laboratories demonstrates their value in identifying this pathogen and usefulness in diagnosing laurel wilt. Recently, Carrillo et al. (4) used the chk and ifw markers to verify the presence of *R. lauricola* in six xyleborine ambrosia beetles other than *X. glabratus*. The new microsatellite-based method reduced the time needed (from 1 week to 1 day) and cost of screening candidate *R. lauricola* isolates, because it eliminates the need for Sanger sequencing.

Despite the detection of the nonspecific amplicons late in the amplification cycles (mean Ct values > 30), the qPCR assay (primers LWD3 and LWD4) is still useful due to the highly sensitive and nearly specific nature of this assay. For example, the assay could be used to assess the rate of spread of *R. lauricola* in different hosts or under different environmental conditions after artificial inoculation, and has been used to detect and quantify *R. lauricola* in a *P. americana* cultivar screening study (29).

The detection limits for the SSU and microsatellite methods were quite different (0.0001 versus 0.1 ng per PCR, respectively). Some of this discrepancy may be due to differences in copy

Table 2. Primer pairs used in the study

Forward primer		Reverse primer		Amplicon size (bp)
Name	Sequence	Name	Sequence	
chk-f	GTTCCACAGCCTGGAAAACC	chk-r	GGTAGAGGACGATGGTTGGC	329–371
ifw-f	TCGAGGTCGTGGACTACAGC	ifw-r	GTGGGACTCGCTGATGAGG	322
LWD1	CCCTGGTGATTCATGATAACTTCT	LWD3	AACGCGTCAAAAGACAACAG	832
LWD4	TTTCTAGGACCGCCGTAATG	LWD3	AACGCGTCAAAAGACAACAG	232

number between the PCR targets. rDNA loci have multiple copies and have been shown to vary (approximately 45 to 150) in some fungi (11,32) while the microsatellite loci are assumed to be low in copy number. The increase in copy number means that less genomic DNA template is required for a PCR to reach the detection limit. This is one drawback of using low-copy loci for detection methods. Still, the difference in copy number does not explain all of the difference in detection limits.

Results with the rDNA SSU primers LWD1 and LWD3 demonstrate problems that can arise when detection of a new pathogen or disease is based on a single locus and undescribed, closely related taxa are present in host tissue. The qPCR primers LWD3 and LWD4 are not taxon specific and, therefore, cannot be used for diagnosing *R. lauricola* and laurel wilt. However, their high sensitivity and ability to quantitatively detect *R. lauricola* in wood samples makes them useful in experimental work with this pathogen (29). In contrast, the microsatellite-based assay is taxon specific. Although it is not sensitive enough to reliably detect the fungus in wood samples (titers of this pathogen are low in diseased tissue; 27), it can be used to quickly screen DNA from cultures for the presence of *R. lauricola*. The microsatellite assay is now used routinely in diagnostic clinics of the University of Florida, and is used by University of Florida researchers to experimentally verify the pathogen's presence in new host trees and ambrosia beetles.

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