Molecular Detection of the Laurel Wilt Fungus, *Raffaelea lauricola*

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Abstract

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The laurel wilt disease fungus, *Raffaelea lauricola*, is killing redbay trees, spreading rapidly in the U.S. southeastern coastal plain forest, and posing a serious threat to the avocado industry in Florida. A molecular tool is urgently required to facilitate detection of this pathogen. The 5' region of the large ribosomal RNA (28S) gene is highly variable among *Raffaelea* spp. and ideal for this purpose but amplification of this sequence from *R. lauricola* has been difficult. Different amplification conditions were tested and a high-fidelity polymerase chain reaction (PCR) procedure utilizing a dNTP mix containing 7-deaza-dGTP was found to reliably amplify 28S sequences from *R. lauricola*. Sequencing the amplified products or cloned inserts also turned out to be difficult and required using a custom-blended sequencing mix containing 1 M betaine, 5% dimethyl sulfoxide, and

dGTP-BigDye v3.1. Three GC-rich stem and loop or cruciform secondary structures were discovered, which may have interfered with amplification. This improved protocol made it possible to partially characterize the internal transcribed spacers sequence from *R. lauricola*, which also has interfering secondary structures. A TaqMan real-time PCR assay was designed using the species-specific 28S sequences and this allowed detection of *R. lauricola* from wood tissues or cultures. Wood tissues from symptomatic redbay, avocado, and sassafras trees in Florida were screened using this TaqMan assay and several were found to test positive for *R. lauricola*. Results were further confirmed by performing Koch's postulates for avocado specimens collected from commercial grooves.

The ambrosia beetle, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), was inadvertently introduced into Savannah, GA in 2002 through infested wooden packing materials that arrived from an unknown Asian destination (6,14). This beetle carries several fungal symbionts, including *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, in its specialized mouth parts (mycangial pouches; 7,9). The adult beetle makes bore holes in the tree trunk, inoculating the wood with *R. lauricola*, which colonizes and causes the laurel wilt disease. Extensive mortality of redbay (*Persea borbonia* (L.) Spreng) and other members of the family Lauraceae is occurring in the coastal plain forest of Georgia, South Carolina, and Florida due to the spread of this beetle and the associated fungus. The disease is now known to affect avocado (*P. americana* Mill) in Florida (12). In addition, camphor (*Cinnamomum camphora* (L.) Sieb) and sassafras (*Sassafras albidum* L.) trees are also dying in Florida due to beetle activities, followed by the inevitable infection by *R. lauricola* (17,18). Redbay trees with laurel wilt were detected recently in Mississippi (15). There have been other reports extending the host and geographic range of the pathogen (Alabama, North Carolina, and so on), most relying on 18S identifications (www.fs.fed.us/r8/forethealth/laurelwilt).

Surveillance is being carried out in Florida to monitor the spread of this disease. When beetle damage (bore holes or beetle frass) is suspected in any avocado trees, molecular diagnosis is often sought to confirm the presence of *R. lauricola* because several *Raffaelea* spp. are known to exist in *X. glabratus* (7,10), and they are often hard to distinguish.

Currently, the genomic DNA extracted from a culture of *Raffaelea* generated from a suspected tree is amplified by a standard polymerase chain reaction (Std-PCR) procedure. A small ribosomal RNA (rRNA) (18S) sequence is amplified and used to confirm

R. lauricola presence (12,15,17,18); however, this marker displays low variability among *Raffaelea* spp. For this reason, taxonomists often emphasize using the highly variable sequences present in the internal transcribed spacers (ITS1 and -2) to detect and identify fungi (19). The problem with ITS amplification from *R. lauricola* was noted early on (6); therefore, the D1/D2 region of the large rRNA (28S) gene has been proposed as the standard for *Raffaelea* identification (1,7,8,10). However, attempts to amplify the highly variable ITS and 28S marker sequences from *R. lauricola* using Std-PCR technique have been difficult. Often, PCR artifacts were generated during these attempts, producing misleading sequence information. This produced lots of false negatives, and even infected trees that produced fungal culture with spore morphology matching *R. lauricola* (9) failed to produce PCR products. A difficult but a successful amplification seems to have allowed detecting only a partial sequence from *R. lauricola* 28S (545 bp) locus (GenBank accession number EU123077) (7). Even though PCR (16) is a popular technique to amplify sequences, failure could still be possible due to (i) trace amounts of plant pathogen DNA present mixed in with abundant plant DNA and (ii) marker sequences containing high GC- or AT-rich regions that produce stem and loop or cruciform secondary structures. Hence, an optimized protocol is urgently required to utilize either the 28S or ITS marker from *R. lauricola* to design a reliable molecular assay. An attempt was made to analyze both markers and design a reliable molecular detection protocol.

Materials and Methods

Specimens. Several suspected redbay, sassafras, avocado, and bay laurel (*Laurus nobilis* L.) trees showing laurel wilt disease symptoms were noticed in different locations of Florida (Table 1). They often showed bore holes in the sap wood due to beetle damage. Beetle frass and stain were noticed. Initially, dead branches appeared but, soon, the affected trees started wilting or died. Samples of wood (approximately 3 to 10 cm) were collected from suspect trees by several pest survey inspectors and submitted for diagnosis. A handheld drill bit was used to bore through the suspected wood and obtain some wood pieces for fungal culturing or DNA extraction. The *R. lauricola* holotype culture C2339 was obtained from Centraalbureau voor Schimmelcultures (The Netherlands) for comparison.

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Culturing. The *Raffaelea* sp. was cultured from infected wood pieces on malt extract agar plates containing streptomycin (0.01%) and cyclohexamide (0.01%) at 30°C in darkness for 10 days (9).

DNA extraction and amplifications. Wood pieces were frozen in liquid nitrogen and finely powdered using a sterile 3-mm glass bead in a Minibeadbeater 96+ (Biospec Products Inc.). A DNeasy Plant Mini Kit (Qiagen Inc.) was used to extract genomic DNA from either cells harvested from a fungal culture plate using sterile HyClone water (5 ml; Thermo Fisher Scientific) or finely powdered wood pieces (100 mg), and eluted using 100 µl of sterile HyClone water. Primers were obtained from a commercial source (Integrated DNA Technologies, Inc.) (Table 2). Std-PCR (16) was performed in a 25-µl reaction volume containing DNA (1 µl), forward and reverse primers (100 pmol; Table 2), buffer (10 mM Tris [pH 8.3], 50 mM potassium chloride, and 1.5 mM magnesium chloride), a dNTP mix (200 µM), and *Taq* DNA polymerase (1 unit; Takara Bio). Std-PCR profile include three linked cycles: (i) 94°C for 2 min (1 cycle); (ii) 94°C for 20 s, 50°C for 18S (varied for other markers), and 72°C for 2 min (35 cycles); and (iii) 72°C for 7 min (1 cycle). High-fidelity (Hf)-PCR (2) was performed in a 50-µl reaction volume containing DNA templates (2 µl), forward and reverse primers (200 pmol; Table 2), buffer (50 mM Tris [pH 9.2], 16 mM ammonium sulfate, and 1.75 mM magnesium chloride), a dNTP mix composed of 350 µM dATP, dTTP, and dCTP plus 87.5 µM dGTP and 262.5 µM 7-deaza-dGTP (Life Technologies-Invitrogen), *Taq* (5 units; Takara Bio), and ACCUZYME (0.5 unit; Bionline Inc.) with a 100-µl mineral oil overlay. Hf-PCR profile included three linked cycles: (i) 94°C for 2 min (1 cycle); (ii) 94°C for 10 s, 45°C for 30 s for 28S or 53°C for 30 s for ITS, and 68°C for 1 min (10 cycles); and (iii) 94°C for 10 s, 45°C for 30 s for 28S or 53°C for 30 s for ITS, and 68°C for 1 min plus 20 s added for every consecutive cycle (20 cycles). A GeneAmp PCR System 9700 (Life Technologies-Applied Biosystems) was used for all amplifications.

Cloning. The PCR products obtained from each sample were cloned into pCR2.1 TOPO by following the manufacturer's recommended procedure (Life Technologies-Invitrogen).

Sequencing. Hf-PCR products (40 µl) were cleaned using a High-Pure column (Roche Diagnostics Corporation) and eluted using sterile HyClone water (40 µl). Sequencing reactions were

performed using Hf-PCR products (4 µl) or plasmids carrying the cloned inserts (100 ng), primer (Table 2), and the BigDye Terminator v3.0 Cycle Sequencing RR-100 mix as suggested by the manufacturer (Life Technologies-Applied Biosystems) or a custom-blended mix containing the dGTP-BigDye Terminator v3.1 Cycle Sequencing RR-100 mix, 5% dimethyl sulfoxide (DMSO; Thermo Fisher Scientific), and 1 M betaine (Sigma-Aldrich). The sequencing reactions were denatured using the BigDye XTerminator purification kit and loaded into an ABI 3130 Genetic Analyzer (Life Technologies-Applied Biosystems). Data collected used ABI Data Collection v3.0 and Sequencing Analysis v5.2 softwares. Sequences with high Phred values (>20) were aligned with the sequences obtained from the GenBank BLAST search in MEGA 5. The sequences were analyzed using mFold software (21) to detect secondary structures.

TaqMan real-time PCR amplifications. Genomic DNA (1 µl) extracted was amplified using 10 pmol each of a forward primer (LW-28S-F1), a reverse primer (LW-28S-R1), and a TaqMan probe (LW-28Sp) (Table 2); and a Takara Ex-Taq master mix (12.5 µl) (Takara Bio) in a 25-µl volume. The amplification profile included (i) 95°C for 30 s (1 cycle) and (ii) 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s (40 cycles). Amplification was performed using a Smart Cycler (Cepheid) and cycle threshold (Ct) values recorded for each sample.

Koch's postulates for laurel wilt disease. If any avocado specimen from a commercial grove tested positive for laurel wilt disease, then Koch's postulates were performed. A small bore hole was made in the trunk of a clean, healthy avocado plant growing in the greenhouse and filled with 100 µl of a fungal spore suspension (approximately 10⁶ spores/ml). The inoculated site was wrapped in parafilm and the plant monitored for 4 weeks. If the inoculated plant died, then a small wood piece was obtained away from the inoculation site and the laurel wilt fungus was cultured as described above. Genomic DNA was extracted from this fungal specimen and tested using TaqMan real-time PCR for *R. lauricola* identification.

Results

Suspected laurel wilt disease-affected redbay, avocado, and bay laurel trees in Florida. Fungal cultures were generated from

Table 1. List of specimens collected from Florida that tested positive for laurel wilt disease^a

Specimen number	Location	Host	Test method
51568	Miami, FL	Avocado	Hf-PCR and sequencing
59989	Miami, FL	Avocado	Hf-PCR and sequencing
66998	Miami, FL	Avocado	TaqMan RT-PCR
71549	Miami, FL	Avocado	TaqMan RT-PCR
72177	Miami, FL	Avocado	TaqMan RT-PCR
72178	Miami, FL	Avocado	TaqMan qRT-PCR
51743	Coral Gables, FL	Bay laurel	Hf-PCR and sequencing
69678	Withalacoochie State Forest (Hernando County), FL	Redbay	TaqMan RT-PCR
71975	Taylor, FL	Redbay	TaqMan RT-PCR
72122	Everglades Swamp (Miami-Dade County), FL	Redbay	TaqMan RT-PCR
72123	Everglades Swamp (Miami-Dade County), FL	Redbay	TaqMan RT-PCR
62611	Gainesville, FL	Sassafras	TaqMan RT-PCR

^a Optimized high-fidelity polymerase chain reaction (Hf-PCR), sequencing, and TaqMan real-time (RT)-PCR procedures were performed.

Table 2. List of primers and probe used for amplification of *Raffaelea lauricola*

Marker	Primer or probe	Sequence	Reference
18S	NS1	5'-GTA GTC ATA TGC TTG TCT C-3'	19
18S	NS4	5'-CTT CCG TCA ATT CCT TTA AG-3'	19
ITS	ITS4	5'-TCC TCC GCT TAT TGA TAT GC-3'	19
ITS	ITS5	5'-GGA AGT AAA AGT CGT AAC AAG G-3'	19
28S	LROR	5'-ACC CGC TGA ACT TAA GC-3'	19
28S	LR3	5'-CCG TGT TTC AAG ACG GG-3'	19
28S	LR5	5'-TCC TGA GGG AAA CTT CG-3'	19
28S	LW28S-F1	5'-CGA GTG AAG CGG CAA CAG CTC A-3'	This study
28S	LW28S-R1	5'-CGC CGC CAG AAG CGT CCT CTC-3'	This study
28S	LW28Sp	(6FAM)-CCG CGG GCC CGA GTT GTA CTT-(BHQ)	This study

the suspected laurel wilt disease samples and their spore morphology (Fig. 1) was found to be very similar to *R. lauricola* (9). Selected fungal cultures from redbay, avocado, and bay laurel were used for molecular analyses to confirm the morphological identification.

Amplification of 18S, 28S, and ITS marker sequences from *R. lauricola* (holotype culture: C2339). A holotype culture of *R. lauricola* (C2339) was first analyzed. Efforts to amplify the 28S and ITS marker sequences from this holotype culture specimen C2339 using the Std-PCR procedure have failed consistently in our hands but amplification of 18S sequence was successful. The 18S sequence (1,035 bp) obtained from the PCR products overlapped and produced 100% identity with the *R. lauricola* sequences existing in the GenBank (FJ514097, GQ996063, GQ329704, and EU170267) in the overlap.

The Hf-PCR procedure has been shown to be at least 10,000-fold more efficient than Std-PCR to amplify fungal marker sequences (3). Hence, the Hf-PCR procedure was tried instead of Std-PCR to amplify the 28S and ITS markers; however, this effort also did not generate any products, indicating that unusual sequences or secondary structures could be interfering with amplification process. Adding 5 or 10% DMSO to the Hf-PCR mix was tried because this reagent was known to weaken secondary structures (20); however, this effort also did not produce any amplified products. Then, a dNTP mix containing 7-deaza-dGTP was tried in the Hf-PCR mix. This modification has worked and did allow amplification of the 28S 5' region (0.9 kb) using the LROR and LR5 primers (Table 2) and the ITS region (0.6 kb) using the ITS4 and ITS5 primers (Table 2). The amplified Hf-PCR products containing 7-deaza-dGTP stained lightly with SYBR Safe dye as expected because the dye does not bind between the 7-deaza-G and C base pairings. Still, a faint DNA band was clearly visible (Fig. 2).

An attempt to sequence these amplified 28S and ITS products using the BigDye Terminator v3.0 sequencing mix was only partially successful. The products were cloned into pCR2.1 TOPO and the plasmid carrying an insert was used as a template for sequencing; however, these attempts also produced only partial success. Interference by unusual sequences or secondary structure in the sequencing reaction was suspected to cause the enzyme to prematurely stop during the extension process. A custom-blend mix containing 1 M betaine, 5% DMSO, and dGTP-BigDye Terminator v3.1 Cycle Sequencing RR-100 mix was tried, and this allowed us to completely sequence the 28S products (932 bp) (GenBank accession number KF515710) from LROR to LR5 primer binding sites but produced only a partial sequence for the 0.6-kb ITS prod-

ucts from the ITS4 primer binding site to the middle of ITS1 spacer (486 bp) (KF515711). A strong secondary structure seems to exist between the partial ITS sequence obtained and the ITS5 primer binding site in the 18S 3' end. Both forward and reverse sequencing reactions failed to resolve this structure.

The sequences obtained were further characterized. The complete 28S sequence (932 bp) was found to overlap a partial *R. lauricola* sequence deposit existing in the GenBank (EU123077) and produced a 100% identity in the overlapped region (545 bp). A BLAST search revealed that the sequences outside the overlap are unique and not represented in the GenBank. The GC content for the entire 932 bp of the 28S fragment is 60% but three segments within this marker have been found to be GC rich: (i) a 55-bp segment from base position number 413 to 467 (87% GC), (ii) a 89-bp segment from base position number 476 to 564 (74% GC), and (iii) a 36-bp segment from base position number 565 to 600 (69% GC),

Fig. 2. High-fidelity polymerase chain reaction amplification of genomic DNA extracted from *Raffaelea lauricola* type culture C2339 using two different dNTP mixes. The 28S-amplified products using a standard dNTP mix containing dGTP (lanes 2–4) and a modified dNTP mix containing 7-deaza-dGTP (lanes 5–7) are shown here. The internal transcribed spacer (ITS)-amplified products using a standard dNTP mix containing dGTP (lanes 8–10) and a modified dNTP mix containing 7-deaza-dGTP (lanes 11–13) are shown here. Lane details: Lane 1, DNA marker; lanes 2, 5, 8, and 11, no DNA control; and lanes 3, 4, 6, 7, 9, 10, 12, and 13, C2339.

Fig. 1. *Raffaelea* culture generated from an avocado tree showing laurel wilt symptoms. **A**, Mycelia developing on a culture plate. **B**, Details of mycelia and spores.

Fig. 3. Mfold-generated secondary structures from three GC-rich segments of the 932-bp amplified *Raffaelea lauricola* 28S sequence. **A**, Segment number 1 (from base position number 413 to 467; 55 bp); **B**, segment number 2 (from base position number 476 to 564; 89 bp); and **C**, segment number 3 (from base position number 565 to 600; 36 bp).

near the LR3 primer (Table 2) binding site. All these GC-rich regions produced strong stem and loop or cruciform secondary structures (Fig. 3) which seem to have interfered with the amplification.

The partial ITS sequence obtained (486 bp) contains an expected partial 28S 5' region (57 bp, 49% GC) from the ITS4 primer binding site, a complete ITS2 spacer (161 bp, 81% GC), a complete 5.8S (154 bp, 49% GC), and a partial ITS1 spacer (114 bp, 62% GC). It appears that a strong secondary structure or a GC-rich region exists between this sequenced segment and the ITS5 primer (Table 2) binding site located in the 18S 3' region. The ITS2 spacer sequence (161 bp) was analyzed and found to contain two strong GC-rich stem and loop structures (*data not shown*); still, the custom sequencing blend was able to resolve them. The ITS1 spacer sequence, on the other hand, could be sequenced only partially (114 bp); a strong secondary structure that exists beyond this 114-bp sequence remains unresolved and their exact sequence is unknown.

Screening symptomatic avocado and bay laurel trees for *R. lauricola*. Genomic DNA obtained from fungal cultures of avocado (specimen numbers 51568 and 59989) and bay laurel (number 51743) were amplified using this optimized protocol and sequenced. The 28S sequences (932 bp) produced 100% identity with those obtained from the *R. lauricola* type culture (C2339). Sequencing the ITS products also produced 100% identity with the type culture (C2339) excluding an ITS1 spacer region containing the strong secondary structure (486 bp). The 18S products obtained from these specimens using Std-PCR also produced 100% identity with the existing *R. lauricola* sequence in the GenBank (FJ514097; 1,035 bp). These findings allowed us to conclude that these three specimens test positive for *R. lauricola*.

A TaqMan real-time PCR assay detects *R. lauricola* from suspected redbay, avocado, and sassafras. In order to design a

<i>Raffaelea</i> Species	GenBank#	28S rRNA Sequence
		87 114
<i>R. albimanens</i>	EU177452	—CCCCCT—GGGGGGGCCCC—GAGTTGTACTT
<i>R. albimanens</i>	EU984296	TCCCCCT—GGGGGGGCCCCGAGTTGTACTT
<i>R. albimanens</i>	HQ688657	—CCCCG—GGGGGGGCCCC—GAGTTGTACTT
<i>R. ambrosiae</i>	EU984297	CTTC—GCGGGGGTCCGAGTTGTAAATT
<i>R. arxii</i>	EU984298	CCCC—GCTGGGGGGCCGAGTTGTAAATT
<i>R. arxii</i>	EU177456	CCCCCGCGGGGGGGTCTGAGTTGTAAATT
<i>R. brunnea</i>	EU177457	CCCCCG—CGGGGGGCCCCGAGTTGTACTT
<i>R. canadensis</i>	EU984299	CCCC—G—CGGGGG—CCCGAGTTGTACTT
<i>R. canadensis</i>	HQ688665	CCCTC—GGGGTCCGCATTGTAAATT
<i>R. castellanii</i>	EU984300	CCCCCGCGGGGGG—CCCGAGTTGTAAATT
<i>R. ellipticospora</i>	HQ688664	CCCCCGCGGGGGGCCCCGAGTTGTAAATT
<i>R. ellipticospora</i>	HQ688663	CCCC—GGGGGGGCCCCGAGTTGTAAATT
<i>R. ellipticospora</i>	HQ688662	CCCC—GGGGGGGCCCCGAGTTGTAAATT
<i>R. ellipticospora</i>	HQ688661	CCCC—GGGGGGGCCCCGAGTTGTAAATT
<i>R. fusca</i>	HQ688658	CTTC—GGGGGGCCGAGTTGTAAATT
<i>R. fusca</i>	EU177449	CCTC—GGGGGGCCCGAGTTGTAAATT
<i>R. gnathotrichi</i>	EU177460	CCCC—GCGGGGGTCCGAGTTGTAAATT
<i>R. motetzi</i>	EU177461	CCCC—GGGGGGCCCGAGTTGTAAATC
<i>R. quercivora</i>	AB552937	CCCC—GGGGGGCCCGAGTTGTAAATC
<i>R. santarosoi</i>	EU984302	CCCCCG—GGGGGGGCCCC—GAGTTGTACTT
<i>R. subalba</i>	EU177443	CCCCT—GGGGGGGCCCC—GAGTTGTAAATT
<i>R. subfusca</i>	HQ688659	CCCT—GGGGGGCCCGAGTTGTAAATT
<i>R. subfusca</i>	EU177473	CCTC—GGGGGGCCCGAGTTGTAAATT
<i>R. sulcati</i>	EU177462	CCCCT—GGGGGGGCCCC—GAGTTGTACTT
<i>R. sulphurea</i>	EU177463	CCCCG—GGGGGTCCCGAGTTGTAAATC
<i>R. sulphurea</i>	HQ688660	CCC—GGGGGGCCCGAGTTGTAAATC
<i>R. tritirachium</i>	EU984303	CCCCT—GGGGGGGTCC—GAGTTGTAAATT
<i>R. tritirachium</i>	EU177464	CCCTT—GGGGGGGTCC—GAGTTGTAAATT
<i>Raffaelea</i> sp.	EU177466	CCC—G—CGGGGG—CCCGAGTTGTACTT
<i>Raffaelea</i> sp.	EU177467	CCCC—GGGGGGGCCCC—GAGTTGTAAATT
<i>R. lauricola</i>	EU177440	CCGC—GGGGGGGCCCCGAGTTGTACTT
Probe LW28Sp		CCGC GGGCCCCGAGTTGTACTT

Fig. 4. *Raffaelea* spp. 28S ribosomal RNA (rRNA) sequences available in the GenBank are aligned using CLUSTAL W and the character positions 87 to 114 displaying high variability are shown here.

TaqMan real-time PCR assay for *R. lauricola*, the 28S rRNA sequence obtained was compared with other *Raffaelea* spp. The 28S rRNA sequences available in the GenBank for *R. albimanens*, *R. ambrosiae*, *R. arxii*, *R. brunnea*, *R. canadensis*, *R. castellanii*, *R. ellipticospora*, *R. fusca*, *R. gnathotrichi*, *R. motetyi*, *R. quercivora*, *R. santorii*, *R. subalba*, *R. subfusca*, *R. sulcati*, *R. sulphurea*, *R. tritirachium*, and *Raffaelea* spp. were obtained and aligned with *R. lauricola* using CLUSTAL W to obtain 566 aligned characters (data not shown). Unique sequences specific to *R. lauricola* were detected and used to design two primers (LW28S-F1 and LW28S-R1; Table 2) and a species-specific probe (LW28Sp; Fig. 4) to amplify a 84-bp segment. A BLAST search performed using this species-specific probe (LW28Sp) sequence (Table 2) has revealed a 100% match to only the *R. lauricola* 28S sequence (EU17440) and not to any other fungal sequences represented in the GenBank, indicating that this sequence is unique and specific. Using this assay, a redbay (Ct value = 18.81, specimen number 71975), an avocado (Ct value = 17.81, specimen number 71549) (Fig. 5), and a sassafras (Ct value = 22.15, specimen number 62611) sample were screened and found to test positive for *R. lauricola*. Additional specimens were screened using this TaqMan assay. Redbay specimens 69678 (Ct value = 16.74), 72122 (Ct value = 22.26), and 72123 (Ct value = 31.87) and avocado specimens 66998 (Ct value = 16.64) and 72177 (Ct value = 27.26) were found to test positive for *R. lauricola*. One more avocado specimen (number 72178) displayed a high Ct value (37.79) but this could be due to low titer of fungal cells in the wood tissue. Fungal cultures were successfully generated from all these specimens (Table 1), and spore morphology was used to confirm their identity as *R. lauricola*. A control healthy, uninfected sample was included in all assays and they always produced a value similar to the no-DNA water control (Ct value > 39). This tool is now routinely deployed to screen all suspected laurel wilt pathogen-affected tree samples received for testing. Over 250 samples have been screened successfully thus far using this TaqMan assay and their infection status determined as infected or healthy. All avocado specimens collected from commercial groves testing positive by molecular diagnosis were further confirmed by performing Koch's postulates by inoculating clean

Fig. 5. TaqMan real-time polymerase chain reaction amplification identifies the presence of *Raffaelea lauricola* from a suspected avocado tree (specimen number 71549). Genomic DNA extracted from a healthy tree and this infected avocado tree tissues were amplified and compared with the *R. lauricola* type culture (C2339) grown on media (positive control) and a no-DNA water control (negative control).

trees, observing their symptoms, successfully recovering the pathogen from inoculated plants, and performing morphological or molecular diagnosis.

Discussion

Amplification of rRNA markers (18S, ITS1-5.8S-ITS2, and 28S) has been routine and often deployed for fungal identification but amplification of 28S and ITS sequences from *R. lauricola* turned out to be very difficult. This was attributed to premature enzyme stopping during the extension process, when *Taq* DNA polymerase encounters unusual sequences (GC- or AT-rich or tandem repeats) or a complex secondary structure (stem and loop and cruciform structures). A combination of Hf-PCR and dNTP mix containing 7-deaza-dGTP has resolved this problem at both 28S and ITS marker loci for *R. lauricola*, and successful amplifications were consistently achieved (Fig. 2). The 7-deaza-dGTP is an analogue that generates Hoogsteen bond formation with C (two hydrogen bonds) instead of the usual Watson-Crick bond formation (three hydrogen bonds). This destabilizes the strong GC-rich stem and loop or cruciform structure and allows *Taq* to successfully complete the extension process (13). The partial 28S sequences (545 bp) obtained earlier by Harrington et al. (9) seems to have succeeded in resolving two secondary structures (Fig. 3A and B) but the third structure (Fig. 3C) present close to the LR3 primer binding site has prevented the enzyme from completing the sequencing reaction.

Incorporation of 7-deaza-dGTP in the PCR products is also known to improve the downstream sequencing reaction (4). However, in the case of *R. lauricola*, sequencing of 28S and ITS products containing 7-deaza-GTP was only partially successful and always failed near the LR3 primer binding site. This prompted a search for additional reagents to improve the sequencing reaction. Two additives, betaine and DMSO, are known to improve amplification through strong secondary structures (4,11,13,20). These reagents facilitate strand separation of double-helix DNA and alter their annealing characteristics. Betaine or DMSO reduces annealing temperature, eliminates spurious GC-hydrogen bonding, and relaxes the secondary structure, resulting in more efficient and specific PCR product synthesis. A dGTP-BigDye Terminator v3.1 sequencing mix was recommended by the manufacturer (Life Technologies and Applied Biosystems) instead of the regular BigDye Terminator v3.0 sequencing mix for templates with strong secondary structures. Adding these three reagents into the sequencing reaction has allowed successful sequencing of the 28S marker products from *R. lauricola* but the ITS marker products still contained a strong structure in the ITS1 spacer that could not be resolved.

The 28S sequences obtained from *R. lauricola* have unique species-specific sequences (Fig. 4), and this allowed the design of a TaqMan real-time PCR probe for detecting laurel wilt from suspect trees. Real-time PCR has some advantages; it allows the screening of more samples and tends to be less labor intensive. Genomic DNA extracted from infected wood tissues could be readily amplified by a TaqMan real-time PCR assay (Fig. 5), eliminating the need to obtain a pure fungal culture for DNA extraction and amplification, and this has further reduced our molecular diagnosis turnaround time. This assay is now routinely deployed to detect and diagnose laurel wilt disease in Florida.

Avocado infection by *R. lauricola* was known (12) but this discovery is now further confirmed by molecular diagnosis and Koch's postulates. This is a cause for concern in Florida (5). Strategies to reduce the spread of this ambrosia beetle (*X. glabratus*) and the fungal pathogen (*R. lauricola*) are urgently required to protect our avocado industry and forest ecosystem.

Acknowledgments

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