

Genetic Variation in Native Populations of the Laurel Wilt Pathogen, *Raffaelea lauricola*, in Taiwan and Japan and the Introduced Population in the United States

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Abstract

Laurel wilt is a vascular wilt disease caused by *Raffaelea lauricola*, a mycangial symbiont of an ambrosia beetle, *Xyleborus glabratus*. The fungus and vector are native to Asia but were apparently introduced to the Savannah, GA, area 15 or more years ago. Laurel wilt has caused widespread mortality on redbay (*Persea borbonia*) and other members of the Lauraceae in the southeastern United States, and the pathogen and vector have spread as far as Texas. Although believed to be a single introduction, there has been no extensive study on genetic variation of *R. lauricola* populations that would suggest a genetic bottleneck in the United States. Ten isolates of *R. lauricola* from Japan, 55 from Taiwan, and 125 from the United States were analyzed with microsatellite and 28S rDNA markers,

and with primers developed for two mating-type genes. The new primers identified isolates as either MAT1 or MAT2 mating types in roughly equal proportions in Taiwan and Japan, where there was also high genetic diversity within populations based on all the markers, suggesting that these populations may have cryptic sex. Aside from a local population near Savannah and a single isolate in Alabama that had unique microsatellite alleles, the U.S. population was genetically uniform and included only the MAT2 mating type, supporting the single introduction hypothesis. This study suggests the importance of preventing a second introduction of *R. lauricola* to the United States, which could introduce the opposite mating type and allow for genetic recombination.

Laurel wilt is a true vascular wilt disease caused by the ambrosia fungus *Raffaelea lauricola* T. C. Harr., Fraedrich, and Aghayeva, which is carried by its ambrosia beetle symbiont, *Xyleborus glabratus* Eichoff (Fraedrich et al. 2008; Harrington et al. 2008). *Raffaelea* is a common genus of saprophytic fungi symbiotically associated with ambrosia beetles (Harrington et al. 2010). *R. lauricola* is unique in that it is the only ambrosia fungus known to cause a vascular wilt. The vector was first detected in the United States in 2002 at Port Wentworth, GA, near Savannah (Rabaglia et al. 2006), where it and its symbiont may have been introduced in solid wood packaging material (Fraedrich et al. 2008). Laurel wilt was first discovered in the United States in 2004, when mortality of redbay (*Persea borbonia*) appeared in the lower coastal plains of South Carolina and Georgia, and in northeastern Florida in 2005 (Fraedrich et al. 2008). Since the first appearance of laurel wilt on redbay and sassafras (*Sassafras albidum*) in South Carolina, Georgia, and Florida, the disease has spread throughout the southeastern United States.

Initial reports in new states were on redbay [Mississippi (Riggins et al. 2010), North Carolina (Hughes et al. 2015), and Texas (Menard et al. 2016)] or on sassafras [Alabama (Bates et al. 2015), Louisiana (Fraedrich et al. 2015a), and Arkansas (Olatinwo et al. 2016)]. However, all tested American Lauraceae have been shown to be susceptible to laurel wilt in inoculation studies (Fraedrich et al. 2008, 2011; Hughes et al. 2013; Ploetz and Konkol 2013). The disease occurs on avocado (*P. americana*), silk bay (*P. humilis*), pondspice (*Listea aestivalis*), and spicebush (*Lindera benzoin*), on the European bay laurel (*Laurus nobilis*), and on the Asian camphortree (*Cinnamomum camphora*) (Fraedrich et al. 2015b, 2016; Hughes et al. 2011, 2012, 2014; Mayfield et al. 2008; Smith et al. 2009a, 2009b). The laurel wilt epidemic is expected to eventually progress to Mexico, a country

rich with native Lauraceae and a large avocado crop industry (Ploetz et al. 2012).

The vector is native to Southeast Asia, most often associated with plant species in the family Lauraceae, and has been reported in India, Bangladesh, Myanmar, China, Japan, and Taiwan (Hulcr and Lou 2013; Rabaglia et al. 2006). *R. lauricola* was consistently isolated from *X. glabratus* in Japan and Taiwan (Harrington et al. 2011), and the pathogen and laurel wilt were recently detected on avocado in Myanmar (Ploetz et al. 2016). *Raffaelea lauricola* is the dominant mycangial symbiont of *X. glabratus* in both the United States and Asia (Campbell et al. 2016; Harrington and Fraedrich 2010; Harrington et al. 2010, 2011). The beetle has been reported to carry up to an estimated 30,000 CFU of *R. lauricola* in the paired oral mycangia of a single beetle, and the overflow of the mycangial growth inoculates the walls of the excavated tunnels in the woody xylem (Harrington and Fraedrich 2010). In both Asia and United States, it is believed that the vector inoculates healthy trees by boring into the xylem, but then aborting the attacks; brood galleries are excavated by later generations of beetles in the wilted branches and stems (Fraedrich et al. 2008, 2015b; Harrington and Fraedrich 2010; Harrington et al. 2011).

It has been assumed that there was a single introduction of the pathogen to the United States (Harrington et al. 2011), but the genetic variation in U.S. and Asian populations has not been extensively studied to test this hypothesis. Harrington et al. (2011) found that some isolates of *R. lauricola* from Japan and Taiwan have a base substitution in the D1/D2 region of the 28S rDNA (LSU) gene. Dreaden et al. (2014) developed two microsatellite markers that amplified trinucleotide repeat regions in the *R. lauricola* genome. The *IFW* microsatellite marker was monomorphic among U.S. isolates, as well as one isolate from Taiwan and another from Japan. The *CHK* microsatellite marker was polymorphic, detecting a second allele (a different number of TCT repeats) in a Japanese isolate (Dreaden et al. 2014).

To test the hypothesis that a single genotype of *R. lauricola* was introduced to the United States from Asia, we analyzed and compared the genetic diversity of Taiwan and Japan populations to that of the U.S. population. If the pathogen is native to Asia but introduced to the United States, we would expect much more diversity

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in Taiwan and Japan than in the United States. To see if the Asian populations may be reproducing sexually, PCR primers for mating-type genes of *R. lauricola* were developed and deployed as genetic markers.

Materials and Methods

Isolates and DNA extraction. Most of the Asian isolates of *R. lauricola* were from *X. glabratus* collected on sticky traps baited with manuka oil lures set out in Fushan, Lienhuachih, and Hsinhsien, Taiwan, in April–December 2009 and in Koshi, Japan, in September 2009 (Harrington et al. 2011). At each of the four sites, two or three sticky traps were in place during a collecting period, but beetles collected from the two or three traps were pooled for each sampling period. Dead beetles were shipped to Iowa, ground and dilution-plated on CSMA (1% malt extract and 1.5% agar, amended with 200 ppm cycloheximide and 100 ppm streptomycin sulfate after autoclaving).

Five additional isolates from Hsinhsien, Taiwan, were obtained in 2014 from a bolt cut from a dying *Cinnamomum osmophloeum* tree that was recently attacked by *X. glabratus*. Three isolates were obtained from three beetles excavated from the bolt using dilution plating, and two isolates were obtained by splitting the bolt and scraping ambrosial growth in a gallery with a flame-sterilized needle and streaking on CSMA.

A total of 125 isolates of *R. lauricola* were collected from 2005 to 2015 in the United States from sapwood of trees with laurel wilt or from adult female *X. glabratus* beetles. Seventeen of the isolates were from *X. glabratus* adults that were trapped in flight, most collected using Lindgren traps baited with manuka oil or cubeb oil (Harrington and Fraedrich 2010). Eighteen isolates were from *X. glabratus* adults reared from naturally infested trees or from bolts taken from healthy trees and baited with manuka oil: one from swampbay (*Persea palustris*), eight from redbay, seven from sassafras, one from avocado, and one from camphortree. Three isolates were from *Xyleborinus saxesenii* beetles excavated from a diseased pondspice bush, and one isolate was from a *Xylosandrus crassiusculus* beetle reared from a sassafras tree with laurel wilt. Isolations from symptomatic sapwood of diseased trees included 58 redbay trees, two *Persea* spp. trees, two avocado trees, 10 sassafras trees, three pondberry (*Lindera melisifolia*) bushes, three camphortrees, three pondspice bushes, and two spicebushes. In total, three isolates were collected from North Carolina, 32 from South Carolina, 55 from Georgia, 11 from Florida, 11 from Alabama, six from Mississippi, two from Louisiana, and five from Texas. The isolates were obtained from the onset of the U.S. epidemic (2005 to 2007) to the recent findings in Texas and Louisiana in 2015.

The 125 U.S. isolates included a special collection of 13 *R. lauricola* isolates obtained from symptomatic sapwood of recently killed redbay trees in 2015 from a small area in coastal Georgia (Halfmoon Landing, Pembroke, St. Simmon's Island, and Jekyll Island). The genetic variation of these 13 isolates was compared with the genetic variation of 16 isolates collected in the same area during the early epidemic (2005 to 2007).

For some isolates, DNA was extracted before storage, but most isolates were previously stored in 15% glycerol at -80°C . For DNA extraction, cultures were plated on MYEA (2% malt extract, 0.2% Difco yeast extract, 1.5% agar) or CSMA and cultured in the dark at room temperature. Mycelium and spores were scraped from the plates and DNA was extracted using a Promega Wizard Kit (Promega, Madison, WI) with cultures 5 to 7 days old or using PrepMan Ultra with cultures 2 to 7 days old.

Large subunit ribosomal (28S) rDNA. The D1/D2 barcoding region of the 28S rDNA gene (Harrington et al. 2010) was sequenced for all studied isolates. In earlier studies (Harrington and Fraedrich 2010; Harrington et al. 2010, 2011), all U.S. isolates had the identical LSU sequence (LSU-A, GenBank Accession No. EU177438), but a single base substitution was detected at position 507 of the 545-bp fragment (LSU-G, HQ688667) in most Asian isolates of *R. lauricola* (Harrington et al. 2011). For identification of the base substitution, PCR was carried out using primers LROR and LR5 (1 μM) with the following program conditions: 85°C for 2 min; followed by

35 cycles of 95°C for 70 s, 58°C for 60 s, and 72°C for 80 s; followed by a final extension of 72°C for 15 min. The PCR products were purified with the GFX PCR DNA and Gel Band Purification Kit and Sanger sequenced with primers LROR and LR3 using a 3730xl DNA Analyzer at the DNA Facility at Iowa State University, Ames.

Small subunit intron ribosomal (18S) rDNA. A 432-bp intron was found to be present in the 18S rDNA (SSU) gene of isolates of *R. lauricola* in the United States (KX958434), and primers were designed to see if all isolates of *R. lauricola* had this intron. Ophiostomatales forward primer SR9Rlaur (5'-CAATTGTCAGAGGT GAAATTCCTTGG-3') is upstream from the intron, and the 3' end of the reverse primer (SR10lrln, 5'-CGCCGCCCTCACTTTTCC-3') is 25 bp into the intron. If the 400-bp PCR product was amplified, the intron was considered present. If no PCR product was amplified, the intron was considered absent. Primer concentration and PCR thermal cycling conditions were as described for the 28S gene.

Microsatellite markers. Two microsatellite markers developed by Dreaden et al. (2014) (*CHK*, GenBank KF381410; and *IFW*, KF381411) were used to identify polymorphisms in the Asian and U.S. populations. The forward primers were fluorescently labeled (Integrated DNA Technologies, Inc., Coralville, IA) with 5' 6-FAM for *CHK* and with 5' HEX for *IFW*. The primer pairs (0.5 μM) were used in separate PCR reactions in a 96-well thermal cycler (PTC-100 MJ Research Inc., Watertown, MA), generally with the following program conditions: 95°C for 5 min; followed by 37 cycles of 95°C for 15 s, 58°C for 15 s, and 68°C for 45 s; with a final extension of 68°C for 5 min. Sizes of the PCR products were determined with a four-capillary Applied Biosystems 3730 DNA Analyzer and Applied Biosystems GeneScan (Thermo Fisher Scientific Inc., Waltham, MA) at the DNA Facility at Iowa State University and Peak Scanner Analysis Software v1.0 (Thermo Fisher Scientific Inc.) using the GS500(-250) setting. To confirm differences among alleles as determined by GeneScan, at least one PCR product representative of each allele was sequenced with the same respective PCR primers but without fluorescent labeling.

Mating-type genes. Primers were developed to amplify a portion of one of the *MAT1-1* genes (*MAT1-1-3*) and a portion of the *MAT1-2* gene (*MAT1-2-1*) of *R. lauricola*. For the *MAT1-1-3* gene, Ophiostomatales primers Oph-MAT1F1 (5'-ATGKCCCRATGARGAYTG CT-3') and Oph-MAT1R2 (5'-GGCGKTKGCRTTGTA YTTGTA-3') designed by Duong et al. (2015) and Arie et al. (1997) thermocycling conditions with a 47°C annealing temperature were used with DNA from *R. lauricola* isolate C2591 from Taiwan. After agarose gel electrophoresis, multiple bands were seen, and the strongest band, about 490 bp in size, was gel-band excised and purified using the GFX PCR DNA and Gel Band Purification Kit. The PCR product was directly sequenced with the same primers. The obtained 481-bp sequence was confirmed to be homologous with the *MAT1-1-3* gene of other Ophiostomatales using BLASTx (Altschul et al. 1990). New primer pairs were designed from this sequence using Oligo v5 (Molecular Biology Insights, Inc., Colorado Springs, CO). Primers LepMAT1F1 (5'-GKCCGATGARGAYTGC-3') and RlrLpt13R (5'-ACCAGGATACATCTGCTTG TG-3') amplified a single 465-bp product with the thermocycling conditions of Arie et al. (1997) and DNA extracted from some, but not all, Asian isolates of *R. lauricola*. The PCR product obtained from Taiwanese isolate C2644 of *R. lauricola* was directly sequenced with the same primers, and the translated BLASTx search with this DNA sequence (KX951454) matched most closely to the predicted protein sequences of *Grossmannia clavigera* mating-type protein 1-1-3 (88% identity, AGH03173), *Leptographium longiclavatum* mating-type protein 1-1-3 (88% identity, AGH03201), the *Ophiostoma querci* mating-type protein 1-1-3 (67% identity, AFD53623) (Fig. 1). The translated sequence from isolate C2644 (KX951454) consisted of 122 amino acid residues and two introns, 52 bp and 46 bp in length, respectively.

In order to identify a marker for the MAT2 mating type, PCR was performed using the degenerate HMG1 and HMG2 primers and thermocycling conditions described by Arie et al. (1997), with a 55°C annealing temperature. A PCR product of 293 bp was obtained with DNA extracted from each of three isolates: C3472 from the United

States; C2589 from Taiwan; and C2661 from Japan. The products were directly sequenced with the same primers and aligned, with no differences among the three DNA sequences. The nucleotide sequence was confirmed to be homologous with the *MAT1-2-1* gene of Ophiostomatales using a BLASTx search. The new internal primers HMGRlrF (5'-TATCGCAAGGACCATCACAAG-3') and HMGRlrR (5'-GTAGCGGTARTNAGGATGAAG-3') were developed and consistently amplified a 260-bp product based on agarose gel electrophoresis, though a faint band directly above the strong 260 bp product was produced in some amplifications. The 260 bp product from isolate C2264 from the United States was directly sequenced with the same primers. A translated BLASTx search with this DNA sequence (KX951454) found that it matched most closely with the predicted protein sequences of *L. terebrantis* HMG mating-type protein 1-2-1 (AGH03134), *G. aurea* HMG mating-type protein 1-2-1 (AGH03141), and *G. clavigera* HMG mating-type protein 1-2-1 (EFX05114) (Fig. 2). The sequence from isolate C2264 was predicted to have 60 amino acid residues and an intron of 78 bp. The

intron begins at a conserved serine position, coinciding with the intron region in *O. querci* and other Ascomycota (Wilken et al. 2012) (Fig. 2).

Two separate PCR reactions with the primers (2 µM) for the respective mating-type genes were run with DNA extracted from each isolate using the following cycling conditions: 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; with a final extension of 72°C for 5 min. The products were examined for the correct band sizes in agarose gels (Fig. 3), and each isolate was classified as MAT1 (*MAT1-1-3* product) or MAT2 (*MAT1-2-1* product). The sizes of the respective PCR products did not vary among the extracted DNA samples using agarose gel electrophoresis.

Analysis of Molecular Variance (AMOVA). Only Asian populations sampled in 2009 were used for AMOVA. Partition of total variance on Euclidean distances was performed in ARLEQUIN 3.5 (Excoffier and Lischer 2010) using the alleles for LSU, *CHK* and *IFW* microsatellite loci, and the idiomorphs of the mating-type locus.

<i>R. lauricola</i>	SDEDCSI IYDTHSKAYRLVPASDVGSIDEQRFRIVGCAKAITTELS---ATASPSKPRIPRPPNAWIIY
<i>G. clavigera</i>	ADDCCILYDAQAKTYRLVPVSDSKFVDLKRFRVMGYARA--SDDG---TTPAP-EPRI PRPPNAWIIY
<i>L. longiclavatum</i>	ADDCCILYDAQAKTYRLVPVSDSKFVDLKRFRVMGYARA--SDDG---TTPAP-EPRI PRPPNAWIIY
<i>O. himal-ulmi</i>	SNEDCSIVYDHQSRTFRLTQMVNANTPCAGRFEIIGHAKAANKEAKNPEPVTTT-ESRI PRPPNAWIIY
<i>O. novo-ulmi</i>	SNEDCSIVYDHQSRTFRLTQMVNASTPCADRFEIIGHAKAANKEAKNPEPVTTT-ESRI PRPPNAWIIY
<i>O. querci</i>	SNEDCSIVYDHQSRTFRLTQMADESTTCASRFEVVGHAKGVNKEVSNPVPTTTSSESRI PRPPNAWIIY
<i>O. montium</i>	SNEDCSIVYDHQSRTFRLTQTADTDVPCPERFEVVGHAKAVVKDDANTTVAGAK-QSRI PRPPNAWIIY
<i>S. schenkii</i>	ADEDCSIVYDSETRTFRLSQMADGEALEAPRYTLVGHAKAV-EDSGDISANATK-SHRI PRPPNAWIIY

<i>R. lauricola</i>	RSQKSKEIRKKIPHATAGYISTLVSQMWKQESPEIRLTYNKAWQAIAHKQMYPG
<i>G. clavigera</i>	RSHKSKEIRKKVPHVTAGYISTLVSQMWKQESCAIRLLYNDKAI EAQKIKHAMYPN
<i>L. longiclavatum</i>	RSHKSKEIRKKVPHVTAGYISTLVSQMWKQESCAIRLLYNDKAI EAQKIKHAMYPN
<i>O. himal-ulmi</i>	RSQKSKEIRKQIPHATAGYISTVVSQMWKLESRETRLCYNSKAI EAQKLHREMYPG
<i>O. novo-ulmi</i>	RSQKSKEIRKQIPHATAGYISTAVSKMWKLESRETRLCYNSKAMEAQKLHREMYPG
<i>O. querci</i>	RSQKSKEIRKQNPATAGFISTAVSKMWKLESREARLRYSKAI EAQKLHREMYPG
<i>O. montium</i>	RSRKSKEIRKQLPHATAGYISTVVSQMWKLESRETRLHYNSKAVEAQKLHKEMYPG
<i>S. schenkii</i>	RSQKSQIRKELPQATAGYISTVVSQMWKLETRETRLLFNAKAVEAQKIHQMY

Fig. 1. Alignment of partial amino acid sequences of the translated *MAT1-1-3* genes of *Raffaelea lauricola* and other Ophiostomatales. Shaded amino acids are conserved across all sequences included in the alignment. The *R. lauricola* sequence was obtained from isolate C2644 from Taiwan. Accession numbers: *R. lauricola* = KX951453; *Grossmannia clavigera* = AGH03173; *Leptographium longiclavatum* = AGH03201, *Ophiostoma himal-ulmi* = AHL24885; *O. novo-ulmi* = ACZ53925; *O. querci* = AFD53623; *O. montium* = AGH03256; *Sporothrix schenkii* = AGG09667.

<i>R. lauricola</i>	YRKDHHKAVKQSNPELSNNEISVILGRQWNYEEDEV RMHY
<i>L. terebrantis</i>	YRKDHHKAVKKS DPELSNNEISVILGRQWNAESDDV RMHY
<i>G. aurea</i>	YRKDHHKAVKKS DPELSNNEISVILGRQWNAESDDV RMHY
<i>G. clavigera</i>	YRKDHHKAVKKS DPELSNNEISVILGRQWNAESDDV RMHY

<i>R. lauricola</i>	HMMAMEIKRQVEKLHPDYRY
<i>L. terebrantis</i>	HTMAIEIKRQVERLHPDYRY
<i>G. aurea</i>	HTMAIEIKRQVERLHPDYRY
<i>G. clavigera</i>	HTMAIEIKRQVERLHPDYRY

Fig. 2. Alignment of the amino acid sequences of the HMG box region of the translated *MAT1-2-1* genes of *Raffaelea lauricola* and other Ophiostomatales. Shaded amino acids are conserved across all sequences included in the alignment. The *R. lauricola* sequence was obtained from isolate C2264 from the United States. Accession numbers: *R. lauricola* sequence = KX951454; *Leptographium terebrantis* = AGH03134; *Grossmannia aurea* = AGH03141; *G. clavigera* = EFX05114.

Variation was determined among sites, among collection dates within a site, and within a collection date at a particular site. The significance of the variance components linked with various levels of genetic structure was tested with nonparametric permutation procedures, which was set to 20,000 permutations.

Pairwise F_{ST} . Comparisons of populations at each collection site in Asia and the U.S. population were made by calculating pairwise F_{ST} with ARLEQUIN 3.5 using the alleles for LSU, *CHK* and *IFW* microsatellite loci, and the mating-type idiomorphs. All 2009 sampling dates were combined for each Asian site (population) in the analysis, except that the Hsinhsien population collected in 2014 was included as a separate population from the 2009 Hsinhsien collections. The significance of genetic difference among populations was tested with nonparametric procedures (1000 permutations).

Genotypic diversity. Every unique combination of alleles among the four genetic markers was considered a unique genotype. Relationships among all genotypes were illustrated by using a matrix of total character differences and UPGMA (unweighted pair group method with arithmetic mean) with 1000 bootstrap replicates in PAUP* v4.0 b10 (Swofford 2002).

Multilocus genotypic diversity was calculated using Stoddart and Taylor's (1988) G with the "poppr" v2.0.2 R package (Kamvar et al. 2014). Following the K-allele model, all alleles were treated as equal to each other, meaning that a mutation from one allele to any other allele is equally probable (Kimura 1983). G values were rarefied (Grünwald et al. 2003; Grünwald and Hoheisel 2006) according to the maximum possible number of genotypes in the smallest population size sampled ($n = 5$) using the poppr v2.0.2 R package (Kamvar et al. 2014) in R v3.2.2.

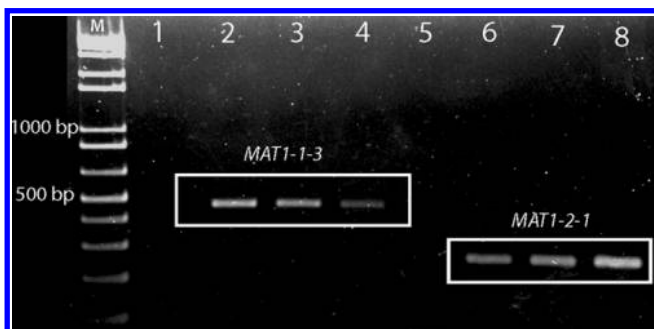


Fig. 3. Agarose gel of PCR products of the partial *MAT1-1-3* (about 500 bp) and *MAT1-2-1* (about 250 bp) genes from six *R. lauricola* isolates, three of each mating type (MAT1 and MAT2, respectively): C2644; C2659; C2636; C2646; C2667; and C2264. The first lane contains the 1 Kb Plus DNA Ladder (Life Technologies, Carlsbad, CA).

Nei's gene diversity (H) (Nei 1987) for the Fushan and Hsinhsien, Taiwan populations, the Koshi, Japan, population, and the U.S. population was calculated with and without clone correction using the poppr v2.0.2 R package.

Linkage disequilibrium. Asian populations were analyzed with and without clone correction for linkage disequilibrium using the index of association (I_A) (Agapow and Burt 2001) in the poppr v2.0.2 R package to test populations for deviation from purely sexual outcrossing. Sampling was set to 999 permutations to test the significance of the I_A value.

Results

In total, 55 isolates from Taiwan (49 from populations), 10 isolates from Japan, and 125 isolates from the United States were analyzed for genetic variation using the D1/D2 region of 28S rDNA, an 18S rDNA intron, the *CHK* and *IFW* microsatellite markers, and the mating-type locus.

Large subunit ribosomal (28S rDNA). Of the 49 isolates of *R. lauricola* collected in 2009 from two sites in Taiwan, 45 had the *LSU-G* allele and four had the *LSU-A* allele, while the 2014 sample from Taiwan included three isolates with the *LSU-G* allele and two isolates with the *LSU-A* allele (Table 1). An isolate from Lienhuachih, Taiwan had the *LSU-G* allele. Of the 10 isolates of *R. lauricola* from Japan, eight had the *LSU-G* allele and two had the *LSU-A* allele (Table 1). Each of the 125 sampled isolates from the United States had the *LSU-A* allele.

Small subunit intron ribosomal (18S rDNA). Although the presence or absence of the intron in the 18S rDNA region was expected to be a source of genetic variation, each of the 125 isolates from the United States, each of the 55 Taiwanese isolates, and each of the 10 Japanese isolates amplified a 400-bp product with the intron-specific primers.

Microsatellite markers. Fourteen different PCR product sizes ranging from 326 to 401 bp and varying by 3 bp increments were detected among the Asian isolates using the *CHK* primers (Table 1). A representative PCR product for each band size was directly sequenced with the PCR primers, and every unique allele had the same number of bp as read by GeneScan, except the 373 bp read by GeneScan was actually 374 bp and the 400 bp read by GeneScan was actually 401 bp. All sequences for *CHK* products had the expected number trinucleotide repeats of TCT. Some infrequent base substitutions occurred in some of the alleles, both inside and outside of the trinucleotide repeat region. The most common allele size in Asia for *CHK* was 329 bp, which was the only allele size found for the *CHK* locus in the U.S. isolates (Table 1). Three *CHK* alleles, including *CHK*-329, were found in both Taiwan and Japan. Four of the *CHK* alleles were found only in Japan, and seven of the *CHK* alleles were found only in Taiwan.

Five different PCR product sizes, which varied by 3-bp increments, were found with the *IFW* microsatellite marker: 306; 312;

Table 1. Alleles for the 28S rDNA (LSU), *CHK* and *IFW* microsatellite markers, and mating type genes found among *Raffaelea lauricola* isolates from two sites in Taiwan, one site in Japan, and across the United States

Country	Location	Year	No. of isolates	Isolates with G or A alleles for 28S rDNA ^a	<i>CHK</i> alleles ^{b,c}	<i>IFW</i> alleles ^{b,c}	Isolates MAT1/MAT2 ^d
Taiwan	Hsinhsien	2009	26	G (24)/A (2)	329 (14), 344 (1), 350 (4), 365 (6), 374 (1)	318 (1), 321 (25)	23/3 ^{**c}
		2014	5	G (3)/A (2)	329 (3), 350 (1), 401 (1)	318 (2), 321 (3)	2/3
	Fushan	2009	23	G (21)/A (2)	NULL (2), 329 (10), 344 (2), 347 (3), 350 (4), 356 (1), 368 (1)	318 (6), 321 (17)	14/9
Japan	Koshi	2009	10	G (8)/A (2)	326 (2), 329 (2), 341 (1), 350 (1), 353 (1), 365 (1), 371 (2)	306 (1), 318 (5), 321 (4)	7/3
USA	All USA	2005-2015	125	G (0)/A (125)	329 (125)	312 (1), 318 (109), 327 (15)	0/125 ^{**}

^a The two 28S rDNA alleles are the dominant Asian (G) allele and the U.S. (A) allele.

^b Allele sizes in bp based on GeneScan readings.

^c Number of isolates with that allele in parentheses.

^d Isolates yielding a *MAT1-1-3* gene PCR product were treated as MAT1, and isolates yielding a *MAT1-2-1* gene PCR product were considered as MAT2.

^e ^{**} Significantly different from the expected 1:1 ratio ($P \leq 0.001$).

318, 321; and 327 bp (Table 1). A representative PCR product was sequenced for each of the five alleles, which revealed a bias in GeneScan results for all the *IFW* alleles by negative 4 bp, that is, based on direct sequencing, the alleles listed above were 310, 316, 322, 325, and 331 bp, respectively. The five microsatellite alleles had varying numbers of trinucleotide repeats of GAC, and there was a small number of base substitutions in some of the alleles, both inside and outside of the repeat region. Only three alleles were found for the *IFW* locus in Asia; the most common were 318 bp and 321 bp, and one isolate in Japan had an allele size of 306 bp (Table 1). The *IFW*-306 allele had one AGC repeat near the 5' end of the PCR product, in addition to the GAC repeats.

The most common *IFW* allele size in the United States was 318 bp, which was also found in Asia. Fifteen isolates from coastal Georgia had the *IFW*-327 allele (Fig. 4, 5), and one isolate from Demopolis in Marengo County, Alabama had the *IFW*-312 allele (Fig. 4, Table 1).

Mating-type genes. All but one of the 190 isolates of *R. lauricola* yielded either a portion of the *MAT1-1-3* gene or a portion of the *MAT1-2-1* gene in PCR analysis (Fig. 3). Only the *MAT1-2-1* mating-type gene product was found among isolates from the United States. Both mating-type genes were found among isolates from Taiwan and Japan (Table 1). Isolate C2688 from Taiwan had positive amplifications for both mating-type markers; it tested positive for the *MAT1-1-3* gene five out of five times, tested as positive for the *MAT1-2-1* gene two out of four times, and had ambiguous results for the *MAT1-2-1* gene two out of four times. Isolate C2688 was perhaps a mixed culture, but it had only single PCR products for the two microsatellite markers. It was categorized as the MAT1 mating type in analyses because the *MAT1-1-3* PCR product was amplified more consistently than the *MAT1-2-1* product.

The ratio of isolates found to have the *MAT1-1-3* product, treated as the MAT1 mating type, to isolates found to have the *MAT1-2-1* product, treated as the MAT2 mating type, was analyzed for deviation from a 1:1 ratio with a Chi-Squared Goodness of Fit test (R statistical package v3.2.2, R Core Team, Vienna). The χ^2 values did not indicate a significant deviation from the expected 1:1 ratio in the 2009 Fushan, 2009 Koshi, and 2014 Hsinhsien populations, but there was a significant deviation from 1:1 in the 2009 Hsinhsien population ($P \leq 0.001$) (Table 1), which was dominated by the GCD1 (*LSU-G*, *CHK*-329, *IFW*-321, *MAT1*) genotype. When this genotype was excluded from the analysis, the 2009 Hsinhsien population still showed a significant deviation from 1:1 ($P \leq 0.05$).

Population structure in Asia. In AMOVA analysis with all four genetic markers, the only significant variation ($P \leq 0.05$) among

isolates from the 2009 Asian populations was found within populations (90%) (Table 2). A small percentage of variation was found among the three individual sites and among the two sampling dates within sites, but these sources of variation were not significant.

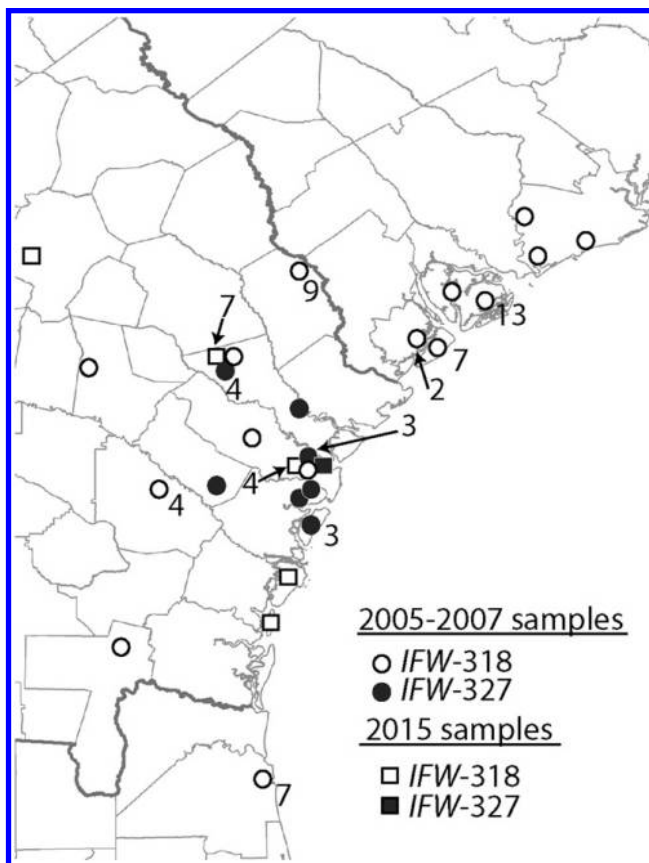


Fig. 5. Distribution of *IFW* microsatellite alleles for *Raffaelea lauricola* isolates collected in South Carolina, Georgia, and northern Florida during the early epidemic (2005 to 2007) and after the epidemic (2015). The two *IFW* alleles found in this region are *IFW*-318 (white fill) and *IFW*-327 (black fill). A circle indicates an isolate was collected between 2005 and 2007, and a square indicates an isolate was collected in 2015. Each symbol represents one isolate, unless indicated otherwise by a number to the right of the symbol or indicated by an arrow.

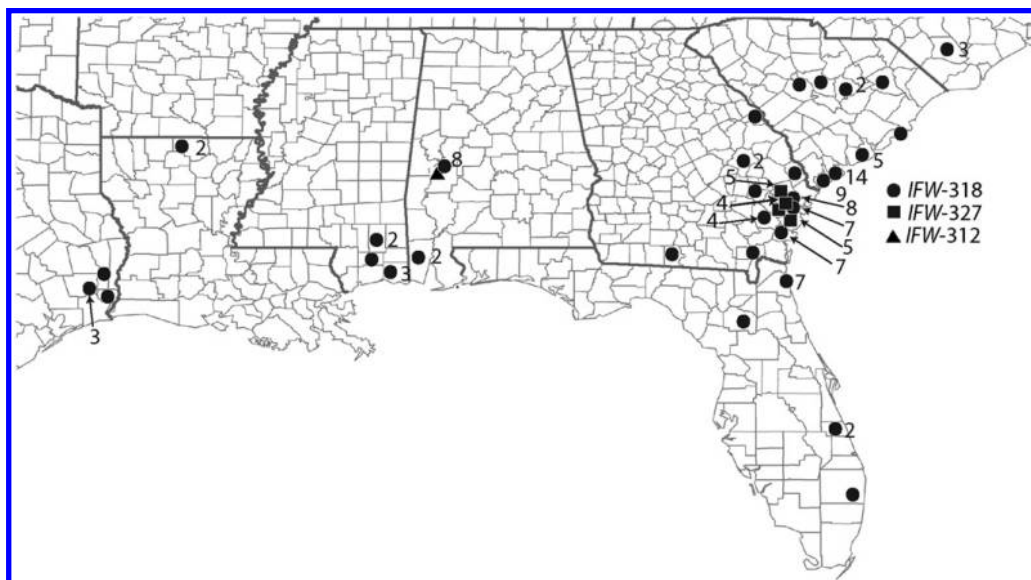


Fig. 4. Distribution of microsatellite *IFW* alleles for *Raffaelea lauricola* isolates collected in the United States. The three *IFW* alleles are *IFW*-318 (circle), *IFW*-327 (square), and *IFW*-312 (triangle). Each symbol represents one isolate, unless indicated otherwise by a number to the right of the symbol or indicated by an arrow.

Because variation among sampling dates was insignificant, data from different sampling dates in 2009 were combined for each respective collection site in other population analyses.

Genetic variation between populations was analyzed with all four genetic markers using the pairwise F_{ST} test. The 2009 Asian populations were all significantly different from each other, except when comparing the Koshi, Japan, population to the Fushan, Taiwan, population (Table 3). In addition, the 2009 and 2014 Hsinhsien populations were significantly different from each other.

Genetic diversity in Asian populations. The four combined marker types resolved 21 genotypes from Asia among the 65 tested isolates. The genotypes were scattered throughout the UPGMA tree based on Nei's genetic distance matrices, with no notable geographic clustering (Fig. 5). Genotype GCD1 was common in each Asian population.

Ten genotypes were found in Fushan among the 19 isolates analyzed, and seven genotypes were found in Japan among the 10 isolates analyzed (Table 4). The genotypic diversity values (G) were relatively high (maximum possible value of 5.0) for each of the Asian populations (Table 4). The 2014 Hsinhsien population (from a single tree) had the highest value (5.0), and the 2009 Hsinhsien population had the lowest value (2.75) among Asian populations. Nei's gene diversity values (H) also were relatively high for the Asian populations (Table 4). The 2014 Hsinhsien population had the highest H value ($= 0.625$). The H value increased greatly for the 2009 Hsinhsien population after clone correction.

Linkage disequilibrium. The linkage disequilibrium analysis for the four genetic markers was performed using index of association (I_A) values to test for random mating in the Taiwan, Japan, and Asia populations overall. The value of I_A should be near zero in a randomly mating population with only sexual reproduction. I_A values were significantly greater than zero without clone correction for Taiwan, Japan, and the Asia population overall ($P \leq 0.01$) (Table 5). Deviation from purely random mating could be explained by dominance of one or a few genotypes within a population due to clonal reproduction. The I_A values were not significantly different from zero with clone correction (only one isolate of each genotype was retained) for Taiwan, Japan, and Asia populations overall (Table 5).

Genotypic diversity in the U.S. population. The 125 U.S. isolates differed at only the *IFW* locus, for which there were only three *IFW* alleles. The U.S. population had a lower G value (1.32) and a lower H value than the Asian populations, with or without clone correction ($H = 0.250$ and 0.0591 , respectively) (Table 4). The most common U.S. genotype was found at both collection sites in Taiwan, but it was not common (Fig. 6). The three U.S. genotypes shared the *LSU-A* variant and *MAT1-2-1* gene with a genotype in Japan, but the Japanese genotype differed at the microsatellite loci (Fig. 6). The pairwise F_{ST} test results using all four markers suggest that the U.S. population was significantly different from all Asian populations ($P \leq 0.01$) (Table 3).

During the early phase of the laurel wilt epidemic (2005-2007), 14 isolates with the rare *IFW*-327 allele were collected in southeastern Georgia (Bryan, Liberty, McIntosh, and Long Counties) (Fig. 5).

In a resampling from 13 wilted redbay trees in Bryan, Liberty, and nearby Glynn counties in 2015, only one isolate (from near Half-moon Landing, Liberty County) had the *IFW*-327 allele, and the other 12 isolates had the *IFW* allele (*IFW*-318) that was dominant throughout the United States (Fig. 5). A χ^2 test revealed that there was a significant difference ($\chi^2 = 143.57$, $P \leq 0.001$) between the ratio of isolates with the *IFW*-327 allele in 2005-2007 (14 with *IFW*-327 allele versus 2 with *IFW*-318 allele) and the ratio of isolates with the same allele in the same region in 2015.

Discussion

The presence of only one mating type in the U.S. population of *R. lauricola* and the near uniformity of microsatellite markers and LSU rDNA sequences strongly suggest that the pathogen population in the United States has gone through a severe genetic bottleneck. This supports the hypothesis that there was a single introduction of *R. lauricola* to the United States. Both mating types of *R. lauricola* were found within the native range of *X. glabratus* in Taiwan and Japan, and there was a much higher level of genetic diversity in the Asian populations than would be expected for a purely asexual species. This is the first indication that a symbiont of an ambrosia beetle has two mating types and may be capable of sexual reproduction.

Substantial genetic diversity was found in Asia, where the *CHK* locus alone had 14 alleles. Pairwise F_{ST} tests indicated some differences among Asian populations, but the AMOVA analysis found that the only significant source of genetic variation was from within populations. Genotypic diversity (G) was high, with the 2014 Hsinhsien, Taiwan, population (from a single tree) having the highest G value. Nei's H values were generally high for Asian populations, with or without clone correction, suggesting greater genetic diversity than would be expected for purely clonal populations.

There are few studies on the genetic diversity of other ambrosia beetle symbionts. Restriction site-associated DNA sequencing was used to analyze genetic diversity and population structure among populations of *R. quercus-mongolicae*, a mycangial symbiont of *Platypus koryoensis* associated with oak mortality in South Korea (Kim et al. 2016). Low genetic diversity suggested that the fungus was introduced to South Korea. Alternatively, *R. quercus-mongolicae* may be asexual. In contrast, Takahashi et al. (2015) reported high genetic diversity in *R. quercivora*, a mycangial symbiont of *P. quercivorus* that is associated with oak mortality in Japan. Three to six genotypes of *R. quercivora* were identified in the mycangia of a single adult, and five to 10 genotypes of *R. quercivora* were detected in individual galleries. Such high genotypic diversity could be associated with an active sexual state. However, Takahashi et al. (2015) used di-nucleotide repeats as markers, and such markers can show a lack of reproducibility compared with trinucleotide markers (Simpson et al. 2013; Steimel et al. 2004).

Most fungal symbionts of ambrosia beetles bud asexually in mycangia, and, until recently it had been thought that all ambrosia fungi only reproduce asexually. However, two *Raffaelea* spp. may form perithecia. Musvuugwa et al. (2015) reported perithecia and ascospores of *R. vaginata* in beetle galleries of a *Lanurgus* sp.

Table 2. Analysis of molecular variance (AMOVA) using four genetic markers of *Raffaelea lauricola* populations isolated from *Xyleborus glabratus* adults trapped at three sites in Asia and up to four sampling dates in 2009

	d.f.	Variance components	Proportion of variance components (%)	P^a
Among sites ^b	2	0.04004	4.69	0.065
Among sampling dates within sites	3	0.04912	5.76	0.203
Within populations	47	0.76404	89.55	0.008
Total	52	0.8532		

^a The P value is for the null hypothesis that there is no significant variation at that level based on 20,022 permutations.

^b The populations were from three sites: Hsinhsien, Taiwan; Fushan, Taiwan; and Koshi, Japan.

Table 3. Pairwise F_{ST} values using four genetic markers between *Raffaelea lauricola* populations from Japan, Taiwan, and the United States

	Japan 2009	Fushan 2009	Hsinhsien 2009	Hsinhsien 2014
Japan 2009				
Fushan 2009	0.01112			
Hsinhsien 2009	0.16413***	0.06466**		
Hsinhsien 2014	-0.0514	0.00439	0.21246*	
USA	0.80322**	0.81058**	0.86767**	0.74146**

^a ** Significant difference between populations ($P \leq 0.01$).

^b Significant difference between populations ($P \leq 0.05$).

(Coleoptera: Curculionidae: Scolytinae: Micracini) in *Olea capensis* in South Africa, but *Lanurgus* spp. are not known to be ambrosia beetles. Davidson (1966) reported perithecia in cultures of *R. seticollis* from galleries of an unidentified ambrosia beetle in hemlock (*Tsuga canadensis*) in New York.

The MAT1 and MAT2 mating types were detected in roughly equal proportions among the *R. lauricola* isolates from Taiwan and Japan. If *R. lauricola* only reproduces asexually in the mycangia as a budding yeast phase and in the galleries as conidia from conidiophores (Fraedrich et al. 2008; Harrington and Fraedrich 2010), then a preponderance of one of the mating types and a high level of linkage disequilibrium might be expected in some populations. However, when corrected for clonal reproduction within a site, the index of association values for the Asia populations did not differ significantly from that expected for randomly mating populations. Although no perithecia have been observed in our numerous attempted crosses of *R. lauricola* isolates of opposite mating type, the 1:1 proportion of mating-type genes and the lack of linkage disequilibrium suggest that there is cryptic sexual reproduction in Asia.

The discovery of MAT1-1 and MAT1-2 idiomorphs among isolates of a fungal species has suggested cryptic sex in other supposedly asexual fungi (Kück and Pöggeler 2009). Although no mating-type genes have been identified earlier in *Raffaelea* spp., mating-type genes have been identified in related genera of Ophiostomatales. The MAT locus and flanking genes have been identified in *Grosmanina* spp. and *Leptographium* spp. (Duong et al. 2012, 2015; Tsui et al. 2013), in *Ophiostoma ulmi*, *O. novo-ulmi*, *O. himal-ulmi*, *O. montium*, and *O. querci* (Jacobi et al. 2010; Paoletti et al. 2005, 2006; Tsui et al. 2013; Wilken et al. 2012), and in *Sporothrix schenckii* and *S. brasiliensis* (Teixeira et al. 2015). The MAT locus has also been partially characterized in *S. globosa* (Kano et al. 2015). Characterization of the MAT locus and development of markers for mating-type genes have demonstrated that many species of *Leptographium* that were thought to be asexual actually have isolates of both mating types and have the potential to sexually reproduce (Duong et al. 2013, 2016). The majority of these *Leptographium* spp. appear to be heterothallic rather than homothallic based on the presence of mating-type genes (Duong et al. 2016).

There are several possible opportunities in the life cycle of *X. glabratus* that could facilitate heterothallic mating of *R. lauricola* in Asia. One hypothesis is that when a mature female *X. glabratus* makes an aborted attack in a healthy tree (Fraedrich et al. 2008, 2015b), the beetle inoculates the xylem with one mating type of the fungus that then systemically colonizes the vascular system. After the tree wilts,

brood attacks by *X. glabratus* are initiated, and the later attacking females may carry a *R. lauricola* strain of opposite mating type. A second possibility is that flightless males of *X. glabratus* may disperse strains of *R. lauricola* to other galleries on the same stem or branch (Kirkendall et al. 1997; Maner et al. 2013). A third opportunity is that one mature female *X. glabratus* could carry both mating types of *R. lauricola* in its mycangia, gut or exoskeleton. Based on our isolates from Japan and Taiwan, a single *X. glabratus* female generally carries one genotype, and therefore one mating type of *R. lauricola* in its mycangia. However, one isolate, C2688 from Taiwan, had both mating-type genes present based on PCR, suggesting that it was a mixed culture of two individual clones of opposite mating type.

In contrast to the genetic diversity of *R. lauricola* populations in Asia, the southeastern U.S. population is nearly uniform and appears to have resulted from an introduction of one or two genotypes (Fraedrich et al. 2008). Shortly after *X. glabratus* was first detected at Port Wentworth, near Savannah, in 2002 (Rabaglia et al. 2006), laurel wilt was noticed 20 km east on Hilton Head, SC, in 2004, and 25 km to the west (Pembroke) and 28 km to the south (Halfmoon Landing) in Georgia in 2005 (Fraedrich et al. 2008). Two alleles for the IFW locus were detected among isolates collected in the Pembroke to Halfmoon Landing area during the early epidemic (2005 to 2007), suggesting that two closely related genotypes of *R. lauricola* were introduced simultaneously. The IFW-327 allele was detected only in this small area west and south of Savannah, where it dominated in the early epidemic. However, a resampling of *R. lauricola* isolates from redbay trees in the same area in 2015 found only one of 13 isolates had the IFW-327 allele. Perhaps the genotype with the IFW-327 allele was less fit and had been replaced by the genotype with the IFW-318 allele, the only genotype that has spread in the United States. The third allele in the United States, IFW-312, was found in a single isolate from a newly discovered outbreak area in Marengo Co., AL, in 2011. It is possible that the IFW-312 genotype was introduced from Asia with the other two genotypes. However, its presence in only one of nine isolates in Alabama and its absence from the earlier collections near Savannah suggest that the IFW-312 allele arose from one random mutation or two stepwise mutations that resulted in the loss of two trinucleotide repeats (Oliveira et al. 2006).

As the epidemic continues to spread, *R. lauricola* has shown the potential to kill most, if not all, American species of Lauraceae (Fraedrich et al. 2008, 2011, 2015b; Hughes et al. 2011, 2012, 2013, 2014; Mayfield et al. 2008; Ploetz and Konkol 2013). Redbay and swampbay have been the primary hosts, although sassafras appears to be a major host for the northward expansion of the epidemic outside the

Table 4. Genetic diversity of *Raffaelea lauricola* populations from Japan, Taiwan, and the United States using four genetic markers

Location	Population	Year	No. Isolates	Nei's Gene Diversity (<i>H</i>)			Genotypic Diversity
				No clone correction	With clone correction	No. of Genotypes/No. of Isolates	
Hsinhsien, Taiwan	HSIN	2009	25	0.274	0.518	8/25	2.75
		2014	5	0.625	0.625	5/5	5.00
Fushan, Taiwan	FUS	2009	19	0.450	0.550	10/19	3.62
Koshi, Japan	JAP	2009	10	0.600	0.607	7/10	4.11
All USA Locations	USA	2005-2015	112	0.059	0.250	3/112	1.32

^a Genotypic diversity with rarefaction using Stoddart and Taylor's (1988) *G*. Values of *G* with rarefaction ranged from 1.0 (one genotype in a population) to 5.0 (each isolate in the population having a different genotype).

Table 5. Index of association (*I_A*) values, without and with clone correction, using genetic markers for 28S rDNA (LSU), *CHK* and *IFW* microsatellite loci, and mating type genes for isolates of *Raffaelea lauricola* from Taiwan and Japan

Country	No. isolates	No. genotypes	<i>I_A</i> (No clone correction)		<i>I_A</i> with clone correction	
			No clone correction	Probability ^a	With clone correction	Probability
Taiwan	55	15	0.387	0.002	-0.0216	0.573
Japan	10	7	0.400	0.009	0.2234	0.408
Total Asia	65	21	0.259	0.003	-0.131	0.954

^a Probability that the index of association value does not differ from a purely sexually outcrossing population.

natural range of redbay (Bates et al. 2015; Fraedrich et al. 2015a; Olatinwo et al. 2016;). Sassafras is highly susceptible to laurel wilt (Fraedrich et al. 2008; Riggins et al. 2011) and is a suitable host for *X. glabratus* (Cameron et al. 2015), but it may be a less attractive host than redbay (Hanula et al. 2008; Mayfield and Hanula 2012). Fraedrich et al. (2015b) suggested that because *X. glabratus* favors some laurel tree hosts over others for brood production, less than ideal brood hosts

could limit further spread of laurel wilt. Other ambrosia beetles rarely carry spores of *R. lauricola* and may introduce them to healthy host trees (Carrillo et al. 2014; Harrington and Fraedrich 2010). However, *X. glabratus* is clearly the primary vector (Carrillo et al. 2014; Harrington et al. 2008, 2011; Harrington and Fraedrich 2010) and appears to have been responsible for spreading the pathogen throughout the Southeast.

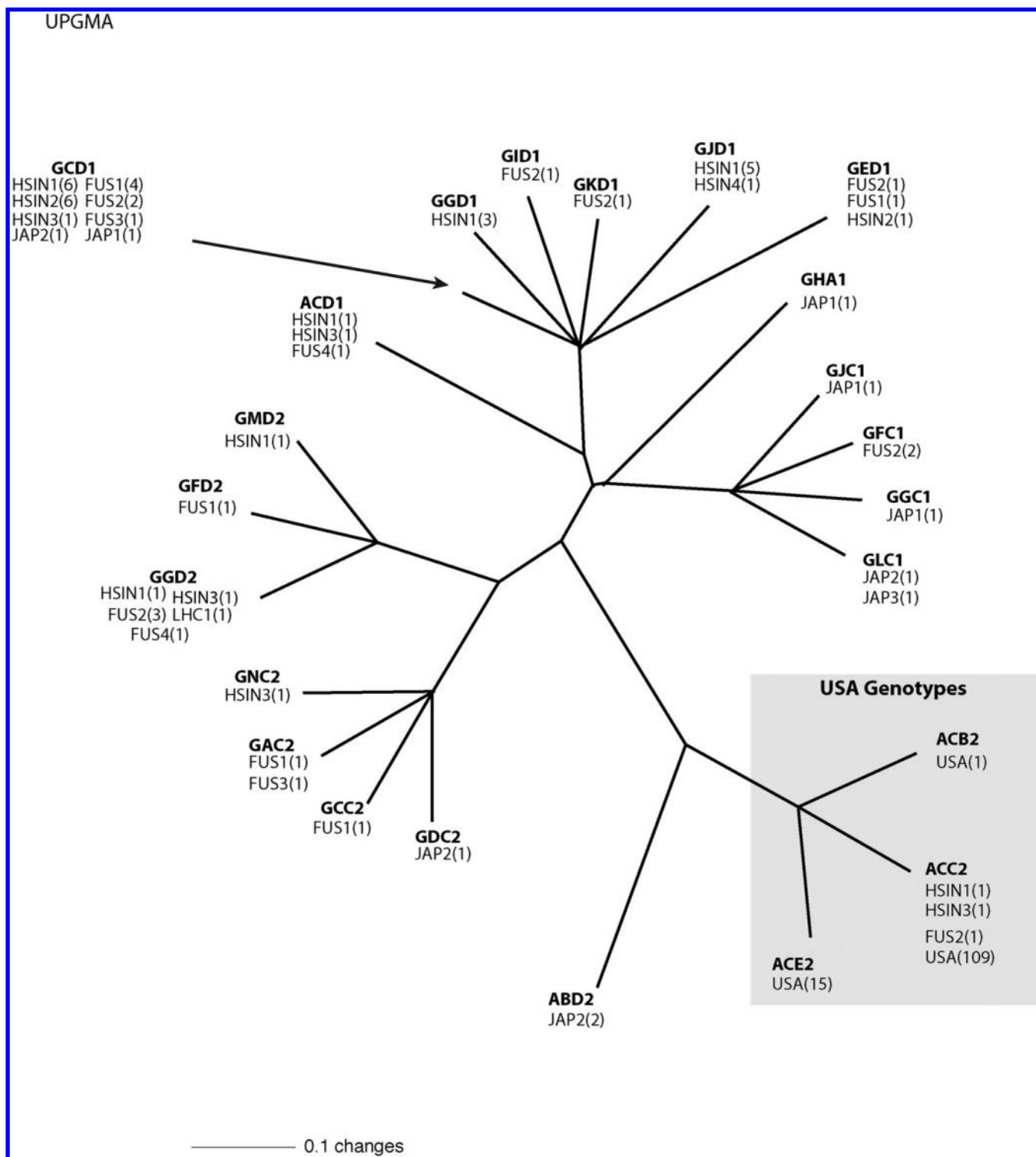


Fig. 6. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram using the standard distance of total character difference of genotypes (bold) of *Raffaelea lauricola* found in Taiwan, Japan, and the United States based on the alleles of 28S rDNA (LSU), *CHK* and *IFW* microsatellite markers, and the mating type locus, respectively. Allele designations are for *LSU* (A or G), *CHK* (A-N), *IFW* (A-E), and mating type gene idiomorphs (1 or 2). Locations are HSIN = Hsinhsien Nursery, Taiwan; FUS = Fushan, Taiwan; LHC = Lienhuachih, Taiwan; and JAP = Koshi, Japan. A number to the right of the location indicates isolates collected on the same date. The number of isolates at a location/collection date with the same genotype is in parentheses. All genotypes found in the United States are enclosed in the gray box. Scale bar indicates genetic distance.

Epidemic populations of introduced pathogens are often dominated by a highly fit genotype (Maynard-Smith et al. 1993), and the laurel wilt epidemic appears to be associated with a single genotype of *R. lauricola*. A similar spread of a single genotype (and one mating type) has been seen in epidemics caused by *O. novo-ulmi*, the cause of Dutch elm disease (DED) (Brasier 2001), a vascular wilt disease similar to laurel wilt. Later in DED epidemics, sexual reproduction apparently occurs through mating with strains of the related *O. ulmi* that carry the opposite mating type (Paoletti et al. 2006). Such hybridizations between species allow for generation of recombinants with new vegetative compatibility groups that reduce the spread of mycoviruses (Brasier 2001; Paoletti et al. 2006), and hybridizations may also create more aggressive strains. Del Sorbo et al. (2000) demonstrated the potential for the oak saprophyte, *O. quercus*, to become a pathogen by artificially inserting the cerato-ulmin gene from *O. novo-ulmi*. The transformed strain caused a vascular wilt disease in elm under laboratory conditions.

This study demonstrates that the *R. lauricola* populations in Asia are highly diverse and that the U.S. population has gone through a severe genetic bottleneck. Both mating types were found in Taiwan and Japan, and high genetic diversity within populations supports the hypothesis that *R. lauricola* is capable of sexual reproduction in Asia. The laurel wilt epidemic in the United States is expanding rapidly in spite of near genetic uniformity, but sexual recombination through a hybridization event or with another introduced strain could lead to an even more aggressive or otherwise fit pathogen. Continued analyses of new *R. lauricola* populations and monitoring ports for *X. glabratus* are both warranted.

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