Genetic variation in native populations of the laurel wilt pathogen, *Raffaelea lauricola*, in Taiwan and Japan and the introduced population in the USA

by

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DEDICATION

I dedicate this thesis to my late mother, Madeline, who finally, and to my surprise, yielded to my request to stop showing my report cards to her friends the week before her last day on Earth. I also dedicate this thesis to my father, Charles, who has always been supportive of all of my life endeavors, and to my step-mother, Jennifer, who has been equally supportive of my ambitions.

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ABSTRACT

Laurel wilt is a true vascular wilt disease caused by *Raffaelea lauricola*, which is a mycangial symbiont of *Xyleborus glabratus*, an ambrosia beetle. The fungus and vector are both native to Asia, but it is believed that both were introduced to the Savannah, Georgia area about 15 years ago. Laurel wilt has caused widespread mortality on redbay (*Persea borbonia*) and other members of the Lauraceae in the southeastern USA, and both pathogen and vector have spread as far as Texas. It is thought that there was a single introduction of *R. lauricola* to the USA, but there are no extensive studies on the genetic variation of *R. lauricola* populations that would suggest a genetic bottleneck in the USA. Ten isolates of *R. lauricola* from Japan, 55 from Taiwan, and 125 from the USA that were collected from *X. glabratus* adults or infected trees 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, indicating these that individuals within 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 USA population was genetically uniform and included only the MAT2 mating type, indicating that the population in the USA has undergone a severe genetic bottleneck. This study suggests the importance of preventing a second introduction of *R. lauricola* to the USA, which could introduce the opposite mating type and allow for genetic recombination and a more aggressive strain of *R. lauricola*.

CHAPTER 1. GENERAL INTRODUCTION

Background

Raffaelea lauricola T. C. Harr, Fraedrich and Aghayeva, the mycangial symbiont of the ambrosia beetle *Xyleborus glabratus* Eichoff, is the only ambrosia fungus known to be a true plant pathogen. *X. glabratus*, native to Southeast Asia (Hulcr and Lou 2013; Rabaglia et al. 2006), was first detected in the USA at Port Wentworth, Georgia in 2002 (Rabaglia et al. 2006). The beetle and the fungal pathogen were presumably introduced near Savannah, Georgia in solid wood packaging material (Fraedrich et al. 2008). In 2004, the vascular wilt disease caused by *R. lauricola*, laurel wilt, was first detected in redbay (*Persea borbonia*) in the lower coastal plains of South Carolina and Georgia near Savannah (Fraedrich et al. 2008), and the disease quickly intensified in this region on redbay and other hosts (Smith et al. 2009a, 2009b). Since 2004, the disease has spread throughout the Southeastern USA on various Lauraceae hosts in Florida (Hughes et al. 2011, 2012, 2014; Mayfield et al. 2008), North Carolina (Hughes et al. 2015), Mississippi (Riggins et al. 2010), Alabama (Bates et al. 2015), Louisiana (Fraedrich et al. 2015a), Texas (Menard et al. 2016), and Arkansas (Olatinwo et al. 2016). Inoculation tests have shown that all American Lauraceae that have been tested are suscepts of laurel wilt (Fraedrich et al. 2008, 2011; Hughes et al. 2013; Peña et al. 2012; Ploetz and Konkol 2013). Laurel wilt is likely to reach Mexico, a country with a large avocado crop industry (Ploetz et al. 2012). The fungus has been recorded in Japan and Taiwan (Harrington et al. 2011), and Myanmar (R. C. Ploetz, Y. Y. Thant, M. A. Hughes, T. J. Dreaden, J. Konkol, A. Kyaw, J. A. Smith, and C. L. Harmon, *unpublished*). While it has been assumed that the USA epidemic is the result of a single introduction (Harrington et al. 2011), there has not been an extensive study on the genetic variation in USA and Asian populations to confirm this hypothesis.

Research on the genetic diversity of the *R. lauricola* population in the USA has indicated that the population is genetically uniform. Harrington et al. (2011) found all DNA sequences of the D1/D2 region of the 28S rDNA (LSU) to be identical in the USA populations, but a single base substitution was found in the LSU gene in most isolates in Taiwan and Japan populations. Dreaden et al. (2014) found all USA isolates tested to be genetically identical using two microsatellite markers that amplified tri-nucleotide base repeat regions in the *R. lauricola* genome. The *IFW* microsatellite marker was monomorphic among USA isolates, as well as one isolate from Taiwan and another from Japan. The *CHK* microsatellite marker was polymorphic, detecting a different allele in the Japan isolate compared to the Taiwan isolate and USA isolates (Dreaden et al. 2014).

The primary goal of this thesis was to determine if the *R. lauricola* population in the USA is the result of a single introduction. This hypothesis was tested by contrasting genetic variation of USA populations of *R. lauricola* with Taiwan and Japan populations. The objectives of this study were to generate markers for two mating type genes of *R. lauricola* and use these as genetic markers in addition to the previously developed LSU, *IFW*, and *CHK* markers to determine the likelihood that *R. lauricola* is capable of sexual reproduction.

Thesis Organization

This thesis consists of three chapters. The first chapter is a general introduction to laurel wilt, followed by a literature review that discusses ambrosia beetle symbiosis, their fungal symbionts and associated plant pathogens, the laurel wilt epidemic, the genetics in *Raffaelea* spp., and mating type genes in Ophiostomatales. The third chapter is a manuscript to be submitted for publication in the journal *Plant Disease*. The manuscript reports on the genetic variation of the

introduced USA population of *R. lauricola* in comparison with native Taiwan and Japan populations. The fourth chapter is a general conclusion derived from the thesis study and proposes future research on *R. lauricola* as well as mating type genes in other *Raffaelea* spp.

Literature Review

Ambrosia beetle symbiosis — Ambrosia beetles are fungal farming and wood boring insects thought to be derived from phloem-feeding bark beetles (Baker 1963). Ambrosia beetles belong to the Scolytidae and Platypodidae within the weevil family, Curculionidae (Beaver 1989). Adult beetles bore into wood and release ambrosia fungi into the tunnels, which then grow in the galleries and provide the primary source of food for all life stages of the ambrosia beetles (Baker 1963; Beaver 1989).

Various researchers, including Berger and Cholodkovskij (1916), Nunberg (1951), and Francke-Grossman (1956), made observations of sac-like organs in adult ambrosia beetles that appeared to harbor fungal spores (Francke-Grossman 1967). These sac-like organs from different beetle species vary in location and structure, but Francke-Grossman (1967) concluded that they are all composed of relatively spacious pouches or tubes possessing secretions that shelter the thin-walled spores of the fungus from desiccation and provide nutrients for fungal growth and survival. Batra (1963) coined the term "mycangia" for these fungal pouches, which are found in most ambrosia beetles and in some bark beetles (Beaver 1989). Setae or clusters of hairs usually aid to secure the fungal spores within the mycangium (Francke-Grosmann 1967; Beaver 1989). The location of the mycangia facilitates the mechanical release of the spores into the galleries, which may be passively aided by the contractions of muscles (Francke-Grossman 1967).

The fungal symbionts are in a yeast-budding phase while growing inside the mycangia (Fraedrich et al. 2008). As the beetles bore into the wood and form tunnels, they farm their fungi by inoculating the sapwood of dead or dying trees with fungal spores (Francke-Grosmann 1967). The fungi growing in the sapwood form conidia on dense clusters of conidiophores (sporodochia), and the larvae and adult beetles feed off of the rich fungal growth (Batra 1967; Harrington 2005). The mycangial sacs of young adult beetles are then inoculated with fungal spores of their symbionts.

Many yeasts appear to have mutualistic relationships with ambrosia beetles (Beaver 1989; Francke-Grosmann 1967). Although the majority of all ambrosia fungi are ascomycetes, basidiomycetous ambrosia fungi are known to be associated with some bark beetles (Beaver 1989; Harrington 2005). Normally there is a dominant ambrosia fungus associated with each ambrosia beetle species, but this fungal species may be considered a component of a larger symbiotic microbial complex, which also includes auxiliary ambrosia fungi and microbes (Batra 1967; Haanstad and Norris 1985).

The majority of described ambrosia fungi fall into either *Raffaelea*, a genus closely related to *Ophiostoma* and *Leptographium*, or *Ambrosiella*, a genus closely related to *Ceratocystis* (Cassar and Blackwell 1996; Harrington et al. 2010a). *Raffaelea* spp. are the most common mycangial symbionts, and *Ambrosiella* spp. tend to have a tighter species-specific association with their ambrosia beetle symbionts (Harrington et al. 2010a, 2014; Mayers et al. 2015). Ambrosia beetles with large, intricate mycangia harbor *Ambrosiella* spp., *Phialophoropsis* spp., or other genera within Ceratocystidaceae (Mayers et al. 2015).

Ambrosia beetles are typically attracted to stressed or dying trees, which may be already colonized by fungi and bacteria (Beaver 1989). The beetles are attracted to tree volatiles such as ethanol, which is a by-product of the metabolism of dying plant tissues and microbes (Beaver 1989). Compared to bark beetles, most ambrosia beetles have a wide host tree range, which may be possible because the host tissue is dead or weakened and the fungal symbiont can grow on sapwood of a wide range of trees (Beaver 1989).

Beetle Symbionts and Plant Pathogens — Raffaelea lauricola is the only ambrosia fungus known to be a true plant pathogen, causing a vascular wilt disease on Lauraceae spp. There are two *Raffaelea* sp. associated with oak (*Quercus* spp.) mortality in Asia, but it is more likely that these oak "wilts" are caused by the beetle associates rather than their fungal partners. *Platypus quercivorus* is known to aggregate in large numbers and mass attack oak trees in Japan, which can result in girdling and death of the trees (Yamasaki et al. 2014). The fungal partner of *P. quercivorus*, *Raffaelea quercivora* (Kubono and Ito 2002), can cause localized lesions in inoculated seedlings but does not cause a vascular wilt disease (Murata et al. 2009). *Raffaelea quercus-mongolicae* is carried by *Playtpus koryoensis*, an ambrosia beetle associated with oak mortality in South Korea (Kim et al. 2009). This beetle species also mass attacks living oak trees (Lee et al. 2011), and the pathogenicity of *R. quercus-mongolicae* has not been elucidated.

Laurel wilt is a vascular wilt disease similar to Dutch elm disease (DED), caused by *Ophiostoma ulmi* and *O. novo-ulmi* (Brasier 2001). These fungi are associates of elm bark beetles in the genus *Scolytus* (Brasier 2001). The majority of DED epidemics are caused by a single aggressive genotype of *O. novo-ulmi,* with just one of the two mating types (Brasier 2001). Over time, mycoviruses infect most clones in the advancing fronts of the epidemic, presumably because the advancing front is of a single vegetative compatibility type, allowing for frequent

fusions of mycelia and spread of the mycovirus (Brasier 2001). Later in the epidemic, sexual reproduction apparently occurs through mating with strains of the related *O. ulmi* of opposite mating type, which allows for the selective acquisition by *O. novo-ulmi* of the *MAT1-1* idiomorph and vegetative compatibility genes from *O. ulmi* (Paoletti et al. 2006). Such hybridizations allow for generation of recombinants with new vegetative compatibility groups and reduced spread of mycoviruses (Brasier 2001; Paoletti et al. 2006).

The potential for closely related species to hybridize and create more aggressive pathogens provides an added threat to multiple introductions of plant pathogens. *Ophiostoma querci* is a widespread oak saprophyte in Europe that is a close relative to *O. novo-ulmi*, and *O. querci* has been found on elm (Brasier 2001). Del Sorbo et al. (2000) demonstrated the potential for *O. querci* to become a pathogen of oak by artificially inserting the cerato-ulmin gene from *O. novo-ulmi* into the genome of *O. querci*. The transformed strain of *O. querci* caused a vascular wilt disease in elm under laboratory conditions (Del Sorbo et al. 2000).

Grosmannia clavigera is the most pathogenic blue-stain fungus associated with the mountain pine beetle (MPB, *Dendroctonus ponderosae*) (Plattner et al. 2008). The fungus appears to thrive under low-oxygen conditions when it is introduced into the water-saturated environment of host pines (Solheim and Krokene 1998; Plattner et al. 2008). As the fungus colonizes and stains the sapwood of pines, *G. clavigera* hinders water and mineral transport in the xylem, somewhat similar to a wilt disease, although the MPB associate does not move systemically in the host (Paine et al. 1997). The fungal growth in the sapwood prevents the tree from efficiently transporting defense compounds to the infection site, which benefits the colonization by MPB (Christiansen et al. 1987; Huber et al. 2004). *Ophiostoma montium* is also

associated with the MPB, but it is not as pathogenic as *G. clavigerum* in inoculation studies (Solheim and Krokene 1998).

Laurel Wilt — Laurel wilt was first discovered in the USA in 2004, when pockets of 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 in Georgia, the disease has spread throughout the Southeastern USA on redbay and various other Lauraceae hosts, including sassafras (*Sassafras albidum*) (Fraedrich et al. 2008; Smith et al. 2009a), as well as avocado (*Persea americana*), pondspice (*Litsea aestivalis*), silk bay (*Persea humilis*), and bay laurel (*Laurus nobilis*) in Florida (Mayfield et al. 2008; Hughes et al. 2011, 2012, 2014), redbay in North Carolina (Hughes et al. 2015), camphor (*Cinnamomum camphora*) in Florida and Georgia (Smith et al. 2009b; Fraedrich et al. 2015b), spicebush (*Lindera benzoin*) in Georgia (Fraedrich et al. 2016), and redbay in Mississippi and Texas (Riggins et al. 2010; Menard et al. 2016). Sassafras has been the main host in recent findings of the disease in Alabama, Louisiana, and Arkansas (Bates et al. 2015; Fraedrich et al. 2015a; Olatinwo et al. 2016). Inoculation studies have shown that all American Lauraceae that have been tested are susceptible to laurel wilt (Fraedrich et al. 2008, 2011; Hughes et al. 2013; Peña et al. 2012; Ploetz and Konkol 2013). Laurel wilt has been detected outside of the USA on avocado in Myanmar (R. C. Ploetz, Y. Y. Thant, M. A. Hughes, T. J. Dreaden, J. Konkol, A. Kyaw, J. A. Smith, and C. L. Harmon, *unpublished*).

Fraedrich et al. (2008) demonstrated that the cause of the redbay mortality was an undescribed *Raffaelea* sp. that is a fungal symbiont of *X. glabratus*. In a field survey conducted by Fraedrich et al. (2008), stems and branches of symptomatic redbay trees possessed streaks of black staining in the sapwood, and the fungus was consistently isolated from this streaking.

Beetle entrance holes were found in stems and branches of diseased trees, and several species of ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) were found in the diseased redbay sapwood, including *Xyleborinus gracilis* Eichoff, *Ambrosiodmus obliquus* LeConte, and *X. glabratus* (Fraedrich et al. 2008). The first two beetles mentioned are indigenous to the Southeastern USA, but *X. glabratus* is an exotic species that was initially reported in the USA at Port Wentworth, Georgia in 2002. *X. glabratus* is native to Southeast Asia, where it is typically associated with plant species in the family Lauraceae (Hulcr and Lou 2013; Rabaglia et al. 2006).

Raffaelea lauricola was described by Harrington et al. (2008). The fungus was likely introduced to the Savannah, Georgia area on solid wood packaging material along with its beetle symbiont, *X. glabratus* (Fraedrich et al. 2008). Ambrosia beetles are typically attracted to alcohols from dead or dying trees. However, *X. glabratus* is unusual in that it is attracted to host plant volatiles from live, healthy trees (Fraedrich et al. 2008; Hanula et al. 2008a; Hanula and Sullivan 2008b). *X. glabratus* possesses paired mandibular mycangial pouches for harboring fungal symbionts, and *R. lauricola* grows in a budding yeast phase within the mycangium (Fraedrich et al. 2008). The conidia of *R. lauricola* are introduced into healthy trees in the process of aborted attacks (Fraedrich et al. 2008). After the pathogen has colonized the xylem, *X. glabratus* females lay eggs in dead or dying trees (Fraedrich et al. 2008). The pathogen travels systemically through the host vessels and causes a lethal vascular wilt disease, comparable to DED, caused by *O. novo-ulmi* (Brasier 2001). In contrast, some researchers believe that tree death is caused by the host overreacting to the pathogen by producing tyloses, leading to vascular dysfunction (Inch et al. 2012).

R. lauricola tolerates cycloheximide, like *Ophiostoma* spp. and other close relatives in the Ophiostomatales (Harrington 1981). The fungus also produces conidiophores and conidia

similar to other ambrosia fungi within *Raffaelea* (Harrington et al. 2008). On agar media, this fungus produces a mucoid growth, which is superficially similar to fungal growth in some other *Raffaelea* spp., such as *R. quercivora* (Kubono and Ito 2002) and *R. quercus-mongolicae* (Kim et al. 2009).

Harrington et al. (2010a) detected a base substitution in the D1/D2 region of the 28S rDNA (LSU) gene sequences of *R. lauricola*. All *R. lauricola* isolates from the USA had identical sequences, but the majority of isolates tested in Taiwan and Japan had a G substituted for an A at the variable position (Harrington et al. 2011). Dreaden et al. (2014) developed two microsatellite markers that amplified tri-nucleotide base repeat regions in the *R. lauricola* genome. The *IFW* microsatellite maker was monomorphic among isolates from the USA, Taiwan, and Japan. The *CHK* microsatellite marker detected two polymorphisms, with the Japanese isolate having 14 more TCT repeats than the Taiwan isolate and USA isolates, which had 10 TCT repeats (Dreaden et al. 2014).

Harrington and Fraedrich (2010b) discovered that up to six *Raffaelea* spp. could be isolated from female adults of *X. glabratus* collected in the USA. *R. lauricola* was isolated from 40 of 41 beetles sampled, and the fungus was isolated up to 30,000 CFU/beetle, the highest CFU of all species isolated. The next most commonly isolated species was *R. subalba* or *R. ellipticospora*, which varied based on the USA population sampled. Other *Raffaelea* spp. that were infrequently isolated include *R. arxii*, *R. fusca*, and *R. subfusca*. These findings suggest that *R. lauricola* grows inside the mycangia with other *Raffaelea* spp., which compete for space and nutrients (Harrington and Fraedrich 2010b). Isolations from *X. glabratus* trapped in Taiwan and Japan also yielded *R. lauricola* in the highest numbers (Harrington et al. 2011). Similar to isolations from *X. glabratus* in the USA, *R. ellipticospora* was the second most frequently

isolated species, and *R. fusca* and *R. subfusca* were infrequently isolated from beetles from Taiwan (Harrington et al. 2011). However, inconsistencies in the mycangial mycoflora of *X. glabratus* in Taiwan, Japan, and the USA suggest that the *X. glabratus* population in the USA came from a different region in Asia (Harrington et al. 2011).

Genetics in Raffaelea — Kim et al. (2016) used restriction-site associated DNA sequencing to analyze genetic diversity and population structure among populations of *R. quercus-mongolicae*, a mycangial symbiont of *P. koryoensis* associated with oak mortality in South Korea (Kim et al. 2009). Low genetic diversity and a lack of population structure for *R. quercus-mongolicae* suggested that the fungus was introduced to South Korea (Kim et al. 2016).

In contrast to *R. quercus-mongolicae*, 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 *P. quercivorus* adult, and five to ten genotypes of *R. quercivora* were detected in individual galleries of *P. quercivorus*. Takahashi et al. (2015) used di-nucleotide repeats as markers, and this type of microsatellite marker may show a lack of reproducibility compared to tri-nucleotide microsatellite markers (Simpson et al. 2013; Steimel et al. 2004). It also is possible that some of the genotypes of *R. quercivora* that Takahashi et al. (2015) detected were not *R. quercivora* but other *Raffaelea* spp. associated with *P. quercivorus*.

Mating Type Genes in Ophiostomatales — Mating compatibility of heterothallic (self-incompatible) ascomycetes is controlled by a single genetic locus in the haploid genome with two mating-type (*MAT*) idiomorphs, designated as *MAT1-1* and *MAT1-2* (Debuchy et al. 2010; Metzenberg and Glass 1990). Sexual reproduction can occur between heterothallic fungi of the same species only when two individuals of the opposite mating type come in contact. In

Sordariomycetes, the *MAT1-1* idiomorph may consist of the *MAT1-1-1* gene with an α1 domain, the *MAT1-1-2* gene, and the *MAT1-1-3* gene with a high-mobility group (HMG) box (Debuchy et al. 2010). The *MAT1-2* idiomorph carries the *MAT1-2-1* gene, which also contains an HMG box (Debuchy et al. 2010).

As of yet, no mating type genes have been identified in *Raffaelea* spp. However, in other genera of Ophiostomatales, the *MAT* locus and flanking genes have been identified in *Grosmannia* spp. (Duong et al. 2012, 2015; Tsui et al. 2013), in *Ophiostoma ulmi*, *O. novo-ulmi*, *O himal-ulmi*, *O. querci*, and *O. montium* (Jacobi et al. 2010; Paoletti et al. 2005, 2006; Tsui et al. 2013; Wilken et al. 2012), and in *Sporothrix schenkii* 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 has indicated that many *Leptographium* spp. that were thought to be asexual have both mating types occurring in nature and have the potential to sexually reproduce (Duong et al. 2013, 2016). Based on the presence of mating type genes, the majority of these *Leptographium* spp. appear to be heterothallic rather than homothallic (Duong et al. 2016).

Most of the fungal symbionts of ambrosia beetles bud asexually in mycangia, and until recently, it had been thought that all ambrosia fungi only asexually reproduce. However, two *Raffaelea* spp. may form perithecia and ascospores. Musvuugwa et al. (2015) reported perithecia and ascospores of *R. vaginata* in beetle galleries of a *Lanurgus* sp. (Coleoptera: Curculionidae: Micracini) in wood of *Olea capensis* in Western Cape Province, South Africa. Davidson (1966) reported perithecia in cultures of *R. seticollis* isolated from ambrosia beetle galleries in hemlock (*Tsuga canadensis*) in New York. However, no mating type gene sequences have been identified

in the genus. If *R. lauricola* can sexually reproduce, there is potential for genetic recombination and generation of new genotypes of greater fitness.

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CHAPTER 2. GENETIC VARIATION IN NATIVE POPULATIONS OF THE LAUREL WILT PATHOGEN, *RAFFAELEA LAURICOLA*, IN TAIWAN AND JAPAN AND THE INTRODUCED POPULATION IN THE USA

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Abstract

Wuest, C. E., Harrington, T. C., Fraedrich, S. W., Yun, H.-Y., and Lu, S.-S. 2016. Genetic variation in native populations of the laurel wilt pathogen, *Raffaelea lauricola*, in Taiwan and Japan and the introduced population in the USA. Plant Dis.

Laurel wilt is a true vascular wilt disease caused by *Raffaelea lauricola*, which is a mycangial symbiont of *Xyleborus glabratus*, an ambrosia beetle. The fungus and vector are both native to Asia, but it is believed that both were introduced to the Savannah, Georgia area about 15 years ago. Laurel wilt has caused widespread mortality on redbay (*Persea borbonia*) and other members of the Lauraceae in the southeastern USA, and both pathogen and vector have spread as far as Texas. It is thought that there was a single introduction of *R. lauricola* to the USA, but there are no extensive studies on the genetic variation of *R. lauricola* populations that would suggest a genetic bottleneck in the USA. Ten isolates of *R. lauricola* from Japan, 55 from Taiwan, and 125 from the USA that were collected from *X. glabratus* adults or infected trees

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, indicating that individuals within thesepopulations may have cryptic sex. Aside from a local population near Savannah and a single isolate in Alabama that had unique microsatellite alleles, the USA population was genetically uniform and included only the MAT2 mating type, indicating that the population in the USA has undergone a severe genetic bottleneck. This study suggests the importance of preventing a second introduction of *R. lauricola* to the USA, which could introduce the opposite mating type and allow for genetic recombination and a more aggressive strain of *R. lauricola*.

Introduction

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 (Harrington et al. 2008). *Raffaelea* is a common genus of saprophytic ambrosia fungi symbiotically associated with ambrosia beetles. However, *Raffaelea lauricola* is a unique species in that it is the only ambrosia fungus known to cause a vascular wilt disease. Laurel wilt was first discovered in the USA in 2004, when mortality of redbay (*Persea borbonia* (L.) Spreng.) appeared in the lower coastal plains of South Carolina and Georgia near Savannah, and in northeastern Florida in 2005 (Fraedrich et al. 2008). Since the first appearance of laurel wilt on redbay and sassafras (*Sassafras albidum* (Nutt.) Nees) (Fraedrich et al. 2008), the disease has spread throughout the Southeastern USA on redbay and various other Lauraceae, including sassafras, avocado (*Persea americana* (Mill.), pondspice (*Listsea aestivalis* (L.) Fernald), silk bay (*Persea humilis*), and bay laurel (*Laurus nobilis*) in Florida (Hughes et al.

2011, 2012, 2014; Mayfield et al. 2008; Smith et al. 2009a), redbay in North Carolina (Hughes et al. 2015), camphor (*Cinnamomum camphora*) in Florida and Georgia (Fraedrich et al. 2015b; Smith et al. 2009b), spicebush (*Lindera benzoin*) in Georgia (Fraedrich et al. 2016), and redbay in Mississippi and Texas (Riggins et al. 2010; Menard et al. 2016). Sassafras has been the main host in recent findings of the disease in Alabama, Louisiana, and Arkansas (Bates et al. 2015; Fraedrich et al. 2015a; Olatinwo et al. 2016). Inoculation studies of American Lauraceae have shown that all that have been tested are susceptible to laurel wilt (Fraedrich et al. 2008, 2011; Hughes et al. 2013; Peña et al. 2012; Ploetz and Konkol 2013). Laurel wilt has been detected outside of the USA on avocado in Myanmar (R. C. Ploetz, Y. Y. Thant, M. A. Hughes, T. J. Dreaden, J. Konkol, A. Kyaw, J. A. Smith, and C. L. Harmon, *unpublished*).The laurel wilt epidemic is expected to eventually progress to Mexico, a country rich with native Lauraceae and a lucrative avocado crop industry (Ploetz et al. 2012). The pathogen and vector are native to Asia, and it has been assumed that the USA epidemic is from a single introduction (Harrington et al. 2011). However, the genetic variation in USA and Asian populations has not been extensively studied (Dreaden et al. 2014; Harrington et al. 2011).

X. glabratus was first detected in the USA at Port Wentworth, GA in 2002 (Rabaglia et al. 2006), and *R. lauricola* was likely introduced to the Savannah, Georgia area on solid wood packaging material along with its symbiont (Fraedrich et al. 2008). *X. glabratus* is native to Southeast Asia, often associated with plant species in the family Lauraceae, and it has been reported in China, India, Bangladesh, Myanmar, Japan, and Taiwan (Hulcr and Lou 2013; Rabaglia et al. 2006). *R. lauricola* is the dominant mycangial symbiont of *X. glabratus* in both the USA and Asia (Harrington et al. 2010a, 2011), and the beetle has been reported to carry up to an estimated 30,000 colony-forming units (CFUs) of this species in the paired mandibular

mycangia of a single beetle (Harrington and Fraedrich 2010b). *X. glabratus* collected in the USA can carry up to six *Raffaelea* spp. inside the mycangia, including *R. ellipticospora*, *R. subalba*, *R. arxii*, *R. fusca*, and *R. subfusca* (Campbell et al. 2016; Harrington and Fraedrich 2010b). The fungal species associated with the mycangia differ somewhat between Asia and the USA (Harrington et al. 2011).

Genetic variation of *R. lauricola* in the USA and Asia has been little studied. 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 sequences. Dreaden et al. (2014) developed two microsatellite markers that amplified tri-nucleotide base repeat regions in the *R. lauricola* genome. The *IFW* microsatellite maker was monomorphic among USA 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 USA, we analyzed and compared the genetic diversity of native Taiwan and Japan populations to that of the USA population. The primary objective of this study was to compare the genetic variation of the Taiwan and Japan populations of *R. lauricola* to the USA population. 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, in addition to the previously developed LSU, *IFW*, and *CHK* markers.

Materials and Methods

Isolates

Most of the Asian isolates of *R. lauricola* were from *X. glabratus* collected on sticky traps baited with manuka oil lures (Harrington et al. 2011). The traps were set up in Fushan, Lienhuachih, and Hsinhsien, Taiwan in Apr-Dec 2009 and Koshi, Japan in Sep 2009. 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). Isolates were stored in 15% glycerol at -80ºC.

Five additional isolates from Hsinhsien, Taiwan were obtained in 2014 from a bolt cut from a stressed *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 the other two isolates were obtained by splitting the bolt and scraping ambrosial growth with a flame-sterilized needle and streaking on CSMA.

A total of 112 isolates of *R. lauricola* were collected from 2005-2015 in the USA 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 or cubeb oil. 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 (*Sassafras albidum*), one from avocado (*Persea americana*), and one from camphortree. Three isolates were from *Xyleborinus saxesenii* beetles excavated from a diseased pondspice (*Litsea aestivalis*) bush, and one isolate was from a *Xylosandrus crassiusculus* beetle reared from a sassafras tree with

laurel wilt. Isolations were also made from symptomatic sapwood from diseased trees, including 45 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 (*Lindera benzoin*). In total, three isolates were collected from North Carolina, 32 from South Carolina, 42 from Georgia, 11 from Florida, two from Louisiana, 11 from Alabama, six from Mississippi, and five from Texas throughout the course of the early epidemic (2005-2007) to the most recently reported findings in Texas and Louisiana in 2015. Isolates were confirmed to be *R. lauricola* based on 28S rDNA (LSU) barcoding (Harrington and Fraedrich 2010b; Harrington et al. 2008, 2010a, 2011) and stored in 15% glycerol at -80ºC.

A special collection of 13 *R. lauricola* isolates were isolated from symptomatic sapwood of recently killed redbay trees in 2015 from Halfmoon Landing, GA, Pembroke, GA, St. Simmon's Island, GA, and Jekyll Island, GA. The genetic variation of these 13 isolates was compared to the genetic variation of 16 isolates collected in the same area during the early epidemic (2005-2007) of laurel wilt.

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. DNA was extracted using a Promega Wizard Kit (Promega, Madison, WI) with cultures 5-7 d old or using PrepMan® Ultra with cultures 2-7 d old.

Large subunit ribosomal (28S) rDNA .

The D1/D2 barcoding region of the 28S rDNA (large subunit, LSU) gene (Harrington et al. 2010a) was sequenced for all studied isolates. In earlier studies (Harrington and Fraedrich

2010b; Harrington et al. 2010a, 2011), all USA isolates had the identical LSU sequence (GenBank Accessions EU177438), but a single base substitution was detected at position 507 of the 545 bp fragment in most Asian isolates of *R. lauricola* (HQ688667) (Harrington et al. 2011). The *LSU*variant dominant in Taiwan and Japan and possessed a G substituted for an A (*LSU-G*) at the variable position. The *LSU-A* variant was less common in Asia and was the only LSU sequence type found in the USA (Harrington et al. 2011). For identification of the base substitution, PCR was carried out using primers LROR and LR5 with the following program conditions: 85ºC for 2 min, followed by 35 cycles of 95ºC for 95 sec, 58ºC for 60 sec, 72ºC for 80 sec, 95ºC for 70 sec, followed by a final extension of 72ºC 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 Sequencing Facility at Iowa State University.

Small subunit intron ribosomal (18S) rDNA.

A 430 bp intron was found to be present in the 18S rDNA (SSU) gene of isolates of *R. lauricola* in the USA, and primers were designed to see if all isolates of *R. lauricola* had this intron. Ophiostomatales forward primer SR9Rlaur (5'-CAATTGTCAGAGGTGAAATTCTTGG-3') was upstream from the intron, and the reverse primer (SR10lrINT, 5'-CGCCGCCCTCACTTTTCC-3') was bound within 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. PCR thermal cycling conditions used were as follows: 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 58ºC for 30 sec, 72ºC for 30 sec, and a final extension at 72ºC for 5 min.

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 USA populations. The forward primers were fluorescently labeled with 5' 6-FAM (Integrated DNA Technologies, Inc., Coralville, IA) for *CHK* and with 5' HEX™ for *IFW*. A fluorescently labeled forward primer and an unlabeled primer flanking each of the respective markers 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: 95ºC for 5 min, followed by 37 cycles of 95ºC for 15 sec, 58ºC for15 sec, 68ºC for 45 sec, with a final extension of 68ºC 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.) 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 determined by GeneScan, a 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 genes (*MAT1-1-3*) and a portion of the *MAT1-2-1* gene. For the *MAT1-1-3* gene, Ophiostomatales primers Oph-MAT1F1 (5'- ATGKCCRATGARGAYTGCT-3') and Oph-MAT1R2 (5'-GGCGKTKGCRTTGTAYTTGTA-3') designed by Duong et al. (2015) and Arie et al.'s (1997) thermocycling conditions with a 47ºC 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; Gish and States 1993). New primer pairs were designed from this sequence using Oligo v5 (Molecular Biology Insights, Inc., Colorado Springs, CO). The new primers were tested for consistent PCR amplification of a single band following the thermocycling conditions of Arie et al. (1997), and primers LepMAT1F1 (5'-GKCCGATGARGAYTGC-3') and RlrLpt13R (5'-ACCAGGATACATCTGCTTGTG-3') consistently amplified a single 465 bp product with DNA extracted from some but not all isolates of *R. lauricola*. The PCR product obtained with these primers and Taiwan isolate C2644 of *R. lauricola* was directly sequenced with the same primers. A translated BLASTx search with this DNA sequence was performed, and the three predicted protein sequences with the highest amino acid identities were the *Grosmannia clavigera* mating type protein 1-1-3 (88% identity, AGH03173), the *Leptographium longiclavatum* mating type protein 1-1-3 (88% identity, AGH03201), and the *Ophiostoma querci* mating type protein 1-1-3 (67% identity, AFD53623) (Fig.1). The translated sequence from isolate C2644 consisted of 122 amino acid residues and two introns, 52 bp and 47 bp in length, respectively, and the putative translation aligned with the *MAT1-1-3* genes of other Ophiostomatales (Fig. 1).

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). A 55ºC annealing temperature was used to amplify the HMG box from the *MAT1-2-1* gene in three isolates of *Raffaelea lauricola*: isolate C3472 from the USA, isolate C2589 from Taiwan, and isolate C2661 from Japan. A PCR product of 293 bp was obtained with DNA

extracted from each of the three isolates. The products were directly sequenced with the same primers and aligned, with no differences found among the three sequences. The nucleotide sequence was confirmed to be homologous with the *MAT1-2-1* gene of Ophiostomatales using a BLASTx search. However, these Arie et al. (1997) primers did not consistently amplify a single PCR product, and new primer pairs were generated and tested for consistent amplification of a single PCR product. Primers HMGRlrF (5'-TATCGCAAGGACCATCACAAG-3') and HMGRlrR (5'- GTAGCGGTARTNAGGATGAAG -3') were chosen for consistent amplification of a 260 bp product. A faint band directly above the strong 260 bp product was produced in some amplifications. The PCR product amplified with these primers and DNA extracted from isolate C2264 from the USA was directly sequenced with the same primers. A translated BLASTx search with this DNA sequence was performed, and the three predicted protein sequences with the highest identities were the *Leptographium terebrantis* HMG mating type protein 1-2-1 (AGH03134), the *Grosmannia aurea* HMG mating type protein 1-2-1 (AGH03141), and the *Grosmannia clavigera* kw1407 HMG mating type protein 1-2-1 (EFX05114), all sharing a 59% identity with the query sequence. 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 several other species in Ascomycota (Wilken et al. 2012), and the exon portion aligned with the homologous HMG boxes specific to the *MAT1-2-1* idiomorph found in other Ophiostomatales (Fig. 2).

Two separate PCR reactions with primers for the respective mating type genes were run with DNA extracted from each isolate using the same thermal cycling conditions as mentioned for the detection of the SSU intron. 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*). Chi-squared goodness-of-fit tests were performed for Asian populations of *R. lauricola* to determine if mating type ratios deviated from the expected 1:1 ratio using the R statistical package v3.2.2 (R Core Team, Vienna).

Analysis of Molecular Variance (AMOVA).

Only Asian isolates collected in 2009 were used for AMOVA. Partition of total variance with AMOVA 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. 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}.

Pairwise population comparisons of each collection site in Asia and the USA population was performed 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 sampling dates were combined for each site in the analysis, except that the Hsinhsien population collected in 2014 was included as a separate population from the 2009 collection. The significance of genetic difference among populations was tested with nonparametric permutation (1000 permutations) procedures.

Genotypic diversity.

Every unique combination of alleles among the four genetic markers was considered a unique genotype. Relationships among all genotypes were analyzed by using the distance of total

character difference matrices 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 *G* (1988) 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, 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.

Nei's gene diversity (*H*) (Nei 1987) for the Fushan and Hsinhsien, Taiwan populations, the Koshi, Japan population, and the USA 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, 10 isolates from Japan, and 125 isolates from the USA were analyzed for genetic variation using the D1/D2 region of 28S rDNA (LSU), the *CHK* and *IFW* microsatellite markers, and the mating type locus.

Large subunit ribosomal (28S rDNA).

The majority of the isolates of *R. lauricola* from the Asian populations had already been surveyed for the two alleles of the D1/D2 region 28S rDNA (Harrington et. al. 2011). Of the 43 isolates of *R. lauricola* collected in 2009 from Taiwan, 39 isolates had the *LSU-G* allele, and four had the *LSU-A* allele (Harrington et al. 2011). Of the 10 isolates of *R. lauricola* from Japan used in this study in 2009, eight isolates had the *LSU-G* allele and two isolates had the *LSU-A* allele (Table 1). Of the five Taiwan isolates collected in 2014, three possessed the *LSU-G* allele, and the other two isolates possessed the *LSU-A* allele. No other variation in LSU sequences was found. Each of the 125 sampled isolates from the USA 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, all isolates from Taiwan, Japan, and the USA amplified a 400 bp product with the intron-specific primers. All 125 isolates from the USA, all 55 Taiwanese isolates, and all 10 Japanese isolates possessed the SSU intron.

Microsatellite markers.

Fourteen different PCR product sizes were detected among the Asian isolates using the *CHK* primers. The sizes of the PCR products ranged from 326 to 400 bp (Table 1). The band sizes varied by three-base increments. A representative PCR product for each band size was directly sequenced with the PCR primers. Sequencing confirmed that almost every unique allele had the same number of bases as read in GeneScan. Two sequenced PCR products had an additional base in the DNA sequence: one sequenced PCR product was 374 bp (but 373 bp in GeneScan analyses) and another was 401 bp (but 400 bp in GeneScan analyses). All sequences

for *CHK* products possessed tri-nucleotide base repeats of TCT, with different allele sizes varying by multiples of three. Some infrequent base substitutions occurred for some of the alleles, both inside and outside of the tri-nucleotide base repeats. 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 USA isolates (Table 1). Of the 14 *CHK* alleles detected, four of the *CHK* alleles were found only in Japan, and seven of the *CHK* alleles were found only in Taiwan. Three *CHK* alleles, including *CHK*-329, were found in both Taiwan and Japan.

Five different PCR product sizes were found with the *IFW* microsatellite marker (Table 1). The sizes of the PCR products based on GeneScan were 306, 312, 318, 321, and 327 bp, which varied by three-base increments. A representative PCR product was sequenced for each of the five alleles. Sequencing 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. Microsatellite allele sequences possessed tri-nucleotide base repeats of GAC, with different allele sizes varying by multiples of three. Based on sequencing, a small number of base substitutions were found 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. The *IFW*-306 allele had one AGC repeat near the 5' end in addition to the GAC repeats in the typical repeat region.

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

Mating type genes.

All but one isolate 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. Both mating type genes were found among isolates from Taiwan and Japan (Table 1). Only the *MAT1-2-1* mating type gene was found among isolates from the USA. Isolate C2688 from Taiwan had positive amplifications for both mating type markers. Using only template from one Wizard extraction, the isolate 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. This isolate was perhaps a mixed culture but categorized as the MAT1 mating type in analyses, because it amplified the *MAT1-1-3* gene more consistently than the *MAT1-2-1* gene.

The ratio of isolates found to have the *MAT1-1-3* gene, treated as the MAT1 mating type, to isolates found to have the *MAT1-2-1* gene, treated as the MAT2 mating type, in Asian populations was analyzed for deviation from a 1:1 ratio (χ^2) with a Chi-Squared Goodness of Fit test. 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 (Table 1). However, the χ^2 values indicated a significant deviation from 1:1 in the 2009 Hsinhsien population ($P \leq 0.001$), 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.

Structure of Asian populations was analyzed with all four genetic markers for significant sources of variation using AMOVA. All significant variation ($P \leq 0.05$) among isolates from the

2009 Asian populations was found within populations (89.55%) (Table 2). A small percentage of variation was found among the three individual sites (4.69%) and among the two sampling dates within sites (5.76%), but these sources of variation were not significant. Because variation among sampling dates was insignificant, data from different sampling dates in 2009 were combined for each respective collection site.

Genetic variation between populations was analyzed with all four genetic markers using the pairwise F_{ST} test. The Asian 2009 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.

Nei's gene diversity values (*H*) were relatively high for the Asian populations (Table 4). The *H* value increased greatly for the 2009 Hsinhsien population with clone correction. The 2014 Hsinhsien population, which comprised five isolates from insects and gallery material from a single tree, had the highest *H* value, with or without clone correction (0.625). The four combined marker types resolved 21 genotypes among the 65 isolates tested from Asia (Fig. 5). Eight genotypes were found in the 2009 Hsinhsien population (26 isolates analyzed), and each of the five isolates from the 2014 Hsinhsien population had different genotypes. Ten genotypes were found in Fushan among the 19 isolates analyzed, and seven genotypes were found in Japan among the nine isolates analyzed (Table 4). Genotype GCD1 was common in every Asian population. The genotypes from Asia were scattered throughout the UPGMA tree based on Nei's genetic distance matrices, with no notable geographic clustering (Fig. 5).

The genotypic diversity values (*G*) were relatively high (maximum possible value of 5.0) for each of the Asian populations (Table 4), with the 2014 Hsinhsien population having the highest value (5.0), and the 2009 Hsinhsien population having the lowest value (2.75) among native populations.

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. When all but one isolate of the GCD1 genotype was removed from each population, I_A values were still significantly greater than zero ($P \leq 0.05$). However, I_A values were not significantly different from zero with clone correction (only one of each genotype was retained) for Taiwan, Japan, and Asia populations overall (Table 5).

Genotypic diversity in the USA population.

The 125 USA isolates differed at only the *IFW* locus, in which there were only three *IFW* alleles. The USA population had the lowest *G* value (1.32) and a lower *H* value than the Asian populations, with or without clone correction (0.250 and 0.0591, respectively) (Table 4). The UPGMA tree grouped the three genotypes found in the USA, and the most common USA genotype was found at both collection sites in Taiwan, but in low numbers (Fig. 6). The three USA genotypes shared the *LSU-A* variant and *MAT1-2-1* gene with a genotype in Japan but

differed at the microsatellite loci (Fig. 6). The pairwise F_{ST} test results using all four markers suggest that the USA 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), a prominent cluster of 14 isolates with the *IFW*-327 allele was collected in southeastern Georgia in Bryan, Liberty, McIntosh, and Long Counties (Fig. 5). In a resampling of Bryan, Liberty, and Glynn Counties in 2015 , only one isolate of out 13 possessed the *IFW*-327 allele, which was collected from a redbay tree near Halfmoon Landing, Liberty County, and the other 12 isolates had the *IFW* allele (*IFW*-318) that is dominant throughout the USA (Fig. 5). A Chi-squared test revealed that there was a significant difference ($\chi^2 = 143.57$, $P \leq 0.001$) between the ratio of isolates found with the *IFW*-327 allele between 2005-2007 (14 with *IFW*-327 allele vs. 2 with *IFW*-318 allele) and the ratio of isolates with the same allele in 2015 (1 with *IFW*-327 allele vs. 12 with *IFW*-318 allele) in this small region.

Discussion

The results of this study support the hypothesis that there was a single introduction of *R. lauricola* to the USA. While only one mating type was found in the USA, two 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 of *R. lauricola* in the Asian populations than would be expected for a purely asexual species. This is the first indication that a mycangial symbiont of an ambrosia beetle has two mating types and may be capable of sexual reproduction. Presence of only one mating type in the USA population of *R. lauricola* and the near uniformity of

microsatellite markers and LSU rDNA sequences strongly suggest that the pathogen population in the USA has gone through a severe genetic bottleneck.

The genetic diversity of *R. lauricola* in Taiwan and Japan is substantial, greater than what would be expected for purely clonal populations. In Asia, the *CHK* locus alone had 14 alleles. Nei's *H* values using all four markers were generally high for Asian populations, with or without clone correction. Genotypic diversity (*G*) was also high, with the 2014 Hsinhsien, Taiwan population having the highest *G* value. This population comprised isolates from two pieces of gallery material and three female beetles excavated from a single section of a naturally infested tree.

In the AMOVA analysis of Asian populations, the only significant source of genetic variation was from within populations, with no significant genetic difference between Taiwan and Japan populations. Given that *R. lauricola* 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 2010b), a high level of linkage disequilibria was expected. However, when corrected for clonal reproduction within a site, the index of association values for the Taiwan, Japan, and Asia overall populations did not differ significantly from that expected for randomly mating populations.

There is a dearth of research on the genetic diversity of *Raffaelea* spp. 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. 2009, 2016). Low genetic diversity and a lack of population structure for *R. quercus-mongolicae* suggested that the fungus was

introduced to South Korea (Kim et al. 2016). Alternatively, *R. quercus-mongolicae* may be native to South Korea and yet have limited diversity because it does not sexually reproduce. In contrast to *R. quercus-mongolicae*, 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 *P. quercivorus* adult, and five to ten genotypes of *R. quercivora* were detected in individual galleries of *P. quercivorus*. Such high genotypic diversity could be associated with an active sexual state. However, Takahashi et al. (2015) used di-nucleotide repeats as markers, and this type of microsatellite marker may show a lack of reproducibility compared to tri-nucleotide microsatellite markers (Simpson et al. 2013; Steimel et al. 2004). Also, ambrosia beetles that carry *Raffaelea* spp. are known to be associated with multiple *Raffaelea* spp. (Batra 1967; Campbell et al. 2016; Funk 1970; Harrington and Fraedrich 2010b; Harrington et al. 2011), and it is possible that some of the genotypes of *R. quercivora* that Takahashi et al. (2015) detected were not *R. quercivora*.

Most of the 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. (Coleoptera: Curculionidae: Micracini) in wood of *Olea capensis* in Western Cape Province, South Africa. Davidson (1966) reported perithecia in cultures of *R. seticollis* isolated from ambrosia beetle galleries in hemlock (*Tsuga canadensis*) in New York.

The MAT1 and MAT2 mating type genes were detected in roughly equal proportions among the *R. lauricola* isolates from Taiwan and Japan. The 2009 Hsinhsien population was the

only population that had a mating type ratio significantly different from the 1:1 ratio that might be expected in a purely random mating population. However, the 2014 Hsinhsien isolations from a single tree (three individual beetles and two pieces of gallery material) yielded isolates of both mating types. Although no perithecia have been observed in our numerous attempted crosses of *R. lauricola* isolates of opposite mating type, the level of gene diversity, lack of linkage disequilibrium, and 1:1 proportion of mating type genes suggest that there is cryptic sexual reproduction in this species in Asia.

The discovery of *MAT1* and *MAT2* idiomorphs (Debuchy et al. 2010) among isolates of a fungal species has suggested cryptic sex in 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 other genera of Ophiostomatales. The *MAT* locus and flanking genes have been identified in *Grosmannia* 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 schenkii* 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 has demonstrated that many species of *Leptographium* that were thought to be asexual have isolates of both mating types and have the potential to sexually reproduce (Duong et al. 2013, 2016). Based on the presence of mating type genes, the majority of these *Leptographium* spp. appear to be heterothallic rather than homothallic (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 laurel tree (Fraedrich et al. 2008; Hughes et al. 2015), the beetle inoculates the xylem with one mating type of the fungus that then colonizes the vascular system. Once *R. lauricola* has sufficiently colonized the sapwood to cause wilt, further brood attacks by *X. glabratus* bores are initiated, and the later attacking females may carry a *R. lauricola* strain of opposite mating type. A second possibility is that male *X. glabratus* may disperse strains of *R. lauricola* to other galleries. The dwarf males first mate with their sisters in the natal galleries (Kirkendall et al. 1997), but then may emerge from the galleries for outbreeding opportunities (Maner et al. 2013). The dwarf males are flightless but can crawl outside of their host tree into a neighboring gallery within the same tree. If a foreign male is permitted entry into a neighboring gallery, potentially carrying spores of *R. lauricola* on his exoskeleton, this would be an opportunity to introduce fungal spores of the opposite mating type. A third opportunity is that one mature female *X. glabratus* could carry both mating types of *R. lauricola* in its mycangia. 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, was an exception to these findings. The PCR results indicated that this isolate had both mating type genes present, perhaps because it was a mixed culture of two individual *R. lauricola* clones of opposite mating type.

In contrast to the diversity of *R. lauricola* populations in Asia, the laurel wilt epidemic in the Southeastern USA appears to have resulted from an introduction of one or two genotypes of *R. lauricola* into the USA around 2002 (Fraedrich et al. 2008), when *X. glabratus* was first detected at Port Wentworth, Georgia (Rabaglia et al. 2006). Laurel wilt was first noticed in

Hilton Head, South Carolina in 2004, and in Pembroke and Halfmoon Landing, Georgia in 2005, each within 65 km of Port Wentworth (Fraedrich et al. 2008). Two alleles for the *IFW* locus were detected among isolates collected early in the epidemic (2005-2007), so it is possible that two closely related genotypes of *R. lauricola* were introduced. The less common *IFW*-327 allele was only detected in a small area south of Savannah, Georgia in the early epidemic. A re-sampling of *R. lauricola* isolates from the same region in 2015 revealed that the ratio of isolates with the *IFW*-327 allele to isolates with the *IFW*-318 allele had decreased substantially since the 2005-2007 sampling, perhaps indicating that the genotype with the *IFW*-318 allele was more fit and had replaced the genotype with the *IFW*-327 allele. Only the *IFW*-318 allele has been found throughout the rest of the epidemic in the Southeastern USA, with the exception of a unique allele, *IFW*-312, found in one of nine isolates collected in Marengo County, Alabama. This new allele may have arisen from one random mutation or two stepwise mutations that resulted in the loss of two tri-nucleotide repeats, as has been found in other microsatellite mutations (Oliveira et al. 2006).

The laurel wilt epidemic has quickly spread across the Southeastern USA from the Savannah area, south to Florida (Mayfield et al. 2008), north to North Carolina (Hughes et al. 2015) and west, reaching Texas (Menard et al. 2016). Redbay and swampbay have been the primary hosts, although sassafras had been recognized early as a major host (Fraedrich et al. 2008; Riggins et al. 2011; Smith et al. 2009a) and appears to be a major host for the expansion of the epidemic outside the natural range of redbay (Bates et al. 2015; Fraedrich et al. 2015a). Even though sassafras is a less attractive host than redbay for *X. glabratus* (Hanula et al. 2008; Mayfield and Hanula 2012), sassafras is highly susceptible to laurel wilt (Riggins et al. 2011) and provides suitable brood material for the beetle (Cameron et al. 2015).

As the epidemic continues to spread, *R. lauricola* has been shown to aggressively colonize and kill most, if not all, American Lauraceae spp. (Fraedrich et al. 2008, 2011, 2015b; Hughes et al. 2011, 2012, 2013, 2014; Mayfield et al. 2008; Ploetz and Konkol 2013). Fraedrich et al. (2015b) suggested that *X. glabratus* favors some laurel tree hosts over others due to their suitability for brood production. When *X. glabratus* reaches an area with hosts that are less ideal for brood production, this could potentially limit or slow the spread of laurel wilt. It has been suggested that other ambrosia beetles may rarely carry spores of *R. lauricola* and introduce them to healthy host trees (Carrillo et al. 2014). However, *X. glabratus* is clearly the primary vector (Carrillo et al. 2014; Harrington et al. 2008, 2011; Harrington and Fraedrich 2010b) and appears to have been responsible for spreading the pathogen throughout the Southeast.

Our observations suggest that the spread of *R. lauricola* in the USA is of a single genotype, which would fit Maynard-Smith et al.'s (1993) epidemic population structure, meaning that sexual reproduction may be frequent in native populations, but sometimes a highly fit individual dominates when a population is introduced to a new location. This individual rapidly reproduces asexually, giving rise to an epidemic clone (Maynard-Smith et al. 1993). A similar spread of a single genotype has been frequently 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. Most epidemics of DED are caused by a single aggressive genotype of *O. novo-ulmi,* with just one of the two mating types (Brasier 2001). Mycoviruses eventually are found in the advancing fronts of the epidemic, presumably because the advancing front is of a single vegetative compatibility type, allowing for frequent fusions of mycelia and spread of the mycovirus. Later in the epidemic, sexual reproduction apparently occurs through mating with strains of the related *O. ulmi* of opposite mating type, which allows for the selective acquisition by *O. novo-ulmi* of

the *MAT1* idiomorph and vegetative compatibility loci from *O. ulmi* (Paoletti et al. 2006). Such hybridizations allow for generation of recombinants with new vegetative compatibility groups and reduced spread of mycoviruses (Brasier 2001; Paoletti et al. 2006).

The potential for closely related species to hybridize and create more aggressive pathogens provides an added threat to multiple introductions of plant pathogens. *Ophiostoma querci* is a widespread oak saprophyte in Europe that is a close relative to *O. novo-ulmi*, and *O. querci* has been found on elm (Brasier 2001). Del Sorbo et al. (2000) demonstrated the potential for *O. querci* to become a pathogen of oak by artificially inserting the cerato-ulmin gene from *O. novo-ulmi* into the genome of *O. querci*. The transformed strain of *O. querci* caused a vascular wilt disease in elm under laboratory conditions (Del Sorbo et al. 2000).

The case of DED and *O. novo-ulmi* differs from that of laurel wilt and *R. lauricola* in the USA in that *R. lauricola* is not known to have mycoviruses to hinder the progression of the epidemic front. Also, the laurel wilt pathogen has spread all across the Southeastern USA with only one mating type, MAT2, and there does not appear to be any closely related species of *Raffaelea* occupying the same niche that could provide an opportunity for species hybridization with a MAT1 strain. As long as there is only one mating type of *R. lauricola* present in the USA, there appears to be limited potential for genetic diversity and development of new genotypes.

This study demonstrates that the *R. lauricola* populations in Asia are highly diverse and that the USA population has gone through a severe genetic bottleneck. The dominant USA genotype was also found in Taiwan. *R. lauricola* and laurel wilt on avocado were reported in Myanmar (R. C. Ploetz, Y. Y. Thant, M. A. Hughes, T. J. Dreaden, J. Konkol, A. Kyaw, J. A. Smith, and C. L. Harmon, *unpublished*), but the pathogen has not been genetically characterized

there. *X. glabratus* is native to several other Asian countries (Hulcr and Lou 2013; Rabaglia et al. 2006), and more data is needed to determine the origin of the introduced USA strain of the fungus. 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. Although laurel wilt caused by *R. lauricola* has already had devastating effects on the Lauraceae in the Southeastern USA, the introduction of the opposite mating type in a second introduction could allow for genetic recombination in the pathogen population. As observed in *O. novo-ulmi* in Europe (Brasier 2001; Paoletti et al. 2006), genetic recombination generates genetic diversity and favors the survival of fitter genotypes. If the opposite mating type of *R. lauricola* were introduced to the USA, or a close relative that could hybridize with *R. lauricola*, the resulting genetic recombination could give rise to a more aggressive strain of *R. lauricola*, creating a more powerful epidemic of laurel wilt as the disease likely spreads south toward Mexico (Menard et al. 2016) and north on sassafras (Bates et al. 2015).

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Tables and Figures

Table 1. All alleles for the 28S rDNA (LSU), *CHK* and *IFW* microsatellite markers, and mating type genes in populations of *Raffaelea lauricola* from Taiwan, Japan, and the USA.

Country	Location	Year	No. of Isolates	Isolates with G or A alleles for 28S rDNA[a]	*CHK* alleles[b,c]	*IFW* alleles[b,c]	Isolates MAT1 or MAT2[d]
Taiwan	Hsinhsien	2009	26	G (24)/A (2)	329 (14), 344 (1), 350 (4), 365 (6), 373 (1)	318 (1), 321 (25)	23/3**[e]
		2014	5	G (3)/A (2)	329 (3), 350 (1), 400 (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 (7)/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 Asian (G) allele and the USA (A) allele.

[b] Allele sizes reported 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 the *MAT1* idiomorph, and isolates yielding a *MAT1-2-1* gene PCR product were considered as the *MAT2* idiomorph.

[e] ** Significantly different from the expected 1:1 ratio ($P \leq 0.001$).

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 20022 permutations.

[b] The populations were from three sites: (1) Hsinhsien, Taiwan; (2) Fushan, Taiwan; (3) Koshi, Japan.

Table 3. Pairwise F_{ST} using four genetic markers among *Raffaelea lauricola* populations from Japan, Taiwan, and USA.

	Japan 2009	Fushan 2009	Hsinhsien 2009	Hsinhsien 2014
Japan 2009				
Fushan 2009	0.01112			
Hsinhsien 2009	0.16413**[a]	0.06466*[b]		
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 ($P \leq 0.05$).

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

				Nei's Gene Diversity (*H*)		Genotypic Diversity	
Location	Population	Year	No. Isolates	No clone correction	With clone correction	No. of Genotypes/ No. of Isolates	Genotypic Diversity (*G*)[a]
Hsinhsien, Taiwan	HSIN	2009	25	0.274	0.518	8/25	2.75
	HSIN	2014	5	0.625	0.625	5/5	5.00
Fushan, Taiwan	FUS	2009	19	0.450	0.550	10/19	3.69
Koshi, Japan	JAP	2009	9	0.500	0.488	7/9	4.28
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 smallest population having a different genotype).

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

			I_A (No clone correction)		I_A with clone correction	
Country	No. Isolates	No. Genotypes	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 does not differ from a purely sexually outcrossing population.

Figure 1. Alignment of a partial sequence of the translated MAT1-1-3 proteins 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*, *Grosmannia clavigera* = AGH03173, *Leptographium longiclavatum* = AGH03201, *Ophiostoma himal-ulmi* = AHL24885, *O. novo-ulmi* = ACZ53925, *O. querci* = AFD53623, *O. montium* = AGH03256, *Sporothrix schenkii* = AGG09667.

Figure 2. Alignment of the HMG box region of the MAT1-2-1 protein 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 USA. Accession numbers: *R. lauricola* sequence, *Leptographium terebrantis* = AGH03134, *Grosmannia aurea* = AGH03141, *G. clavigera* kw1407 = EFX05114.

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

Figure 4. Distribution of microsatellite *IFW* alleles for *Raffaelea lauricola* isolates collected in the USA . 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.

Figure 5. Distribution of *IFW* microsatellite alleles for *Raffaelea lauricola* isolates collected in South Carolina, Georgia, and northern Florida during the early epidemic (2005-2007) 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 symbol indicates an isolate was collected between 2005-2007, and a square symbol 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.

Figure 6. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram using and the standard distance of total character difference of genotypes (bold) of *Raffaelea lauricola* found in Taiwan, Japan, and the USA 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; JAP = Koshi, Japan. A number to the right of the location indicates isolates that share a collection date. The number of isolates with a certain genotype at a location/collection date is in parentheses. All genotypes found in the USA are enclosed in the gray box. Scale bar indicates genetic distance.

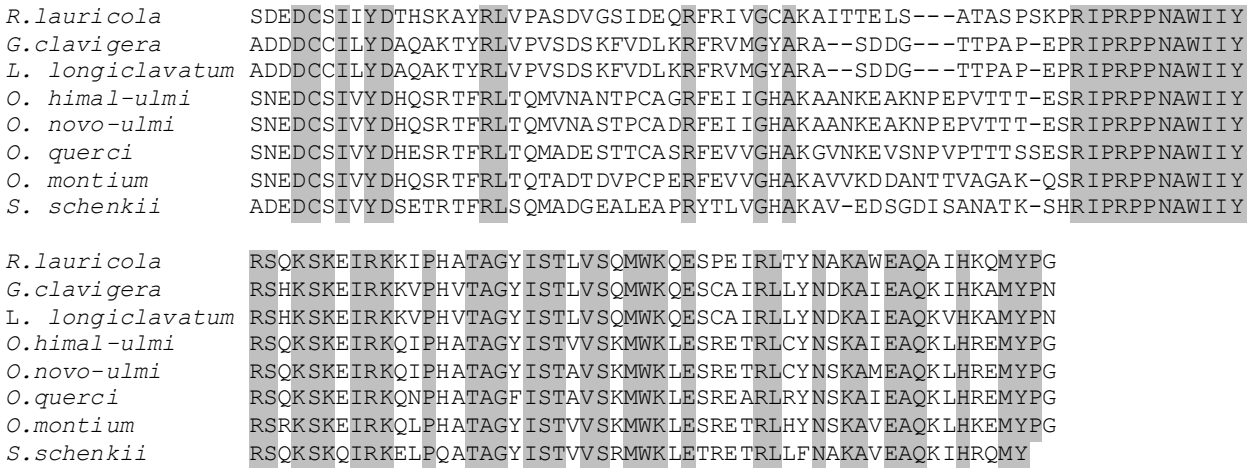
R.lauricola SDEDCSIIYDTHSKAYRLVPASDVGSIDEQRFRIVGCAKAITTELS---ATASPSKPRIPRPPNAWIIY
G.clavigera ADDDCCILYDAQAKTYRLVPVSDSKFVDLKRFRVMGYARA--SDDG---TTPAP-EPRIPRPPNAWIIY
L. longiclavatum ADDDCCILYDAQAKTYRLVPVSDSKFVDLKRFRVMGYARA--SDDG---TTPAP-EPRIPRPPNAWIIY
O. himal-ulmi SNEDCSIVYDHQSRTFRLTQMVNANTPCAGRFEIIGHAKAANKEAKNPEPVTTT-ESRIPRPPNAWIIY
O. novo-ulmi SNEDCSIVYDHQSRTFRLTQMVNASTPCADRFEIIGHAKAANKEAKNPEPVTTT-ESRIPRPPNAWIIY
O. querci SNEDCSIVYDHESRTFRLTQMADESTTCASRFEVVGHAKGVNKEVSNPVPTTTSSESRIPRPPNAWIIY
O. montium SNEDCSIVYDHQSRTFRLTQTADTDVPCPERFEVVGHAKAVVKDDANTTVAGAK-QSRIPRPPNAWIIY
S. schenkii ADEDCSIVYDSETRTFRLSQMADGEALEAPRYTLVGHAKAV-EDSGDISANATK-SHRIPRPPNAWIIY
R.lauricola RSQKSKEIRKKIPHATAGYISTLVSQMWKQESPEIRLTYNAKAWEAQAIHKQMYPG
G.clavigera RSHKSKEIRKKVPHVTAGYISTLVSQMWKQESCAIRLLYNDKAIEAQKIHKAMYPN
L. longiclavatum RSHKSKEIRKKVPHVTAGYISTLVSQMWKQESCAIRLLYNDKAIEAQKVHKAMYPN
O.himal-ulmi RSQKSKEIRKQIPHATAGYISTVVSKMWKLESRETRLCYNSKAIEAQKLHREMYPG
O.novo-ulmi RSQKSKEIRKQIPHATAGYISTAVSKMWKLESRETRLCYNSKAMEAQKLHREMYPG
O.querci RSQKSKEIRKQNPHATAGFISTAVSKMWKLESREARLRYNSKAIEAQKLHREMYPG
O.montium RSRKSKEIRKQLPHATAGYISTVVSKMWKLESRETRLHYNSKAVEAQKLHKEMYPG
S.schenkii RSQKSKQIRKELPQATAGYISTVVSRMWKLETRETRLLFNAKAVEAQKIHRQMY

Figure 1

```
R. lauricola    YRKDHHKAVKQSNPELSNNEISVILGRQWNYEEDEVRMHY
L. terebrantis YRKDHHKAVKKSDPELSNNEISVILGRQWNAESDDVRMHY
G. aurea        YRKDHHKAVKKSDPELSNNEISVILGRQWNAESDDVRMHY
G. clavigera    YRKDHHKAVKKSDPELSNNEISVILGRQWNAESDDVRMHY

R. lauricola    HMMAMEIKRQVEKLHPDYRY
L. terebrantis HTMAIEIKRQVERLHPDYRY
G. aurea        HTMAIEIKRQVERLHPDYRY
G. clavigera    HTMAIEIKRQVERLHPDYRY
```

Figure 2

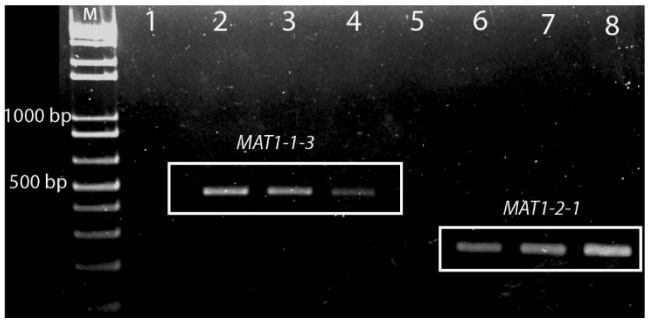

Figure 3

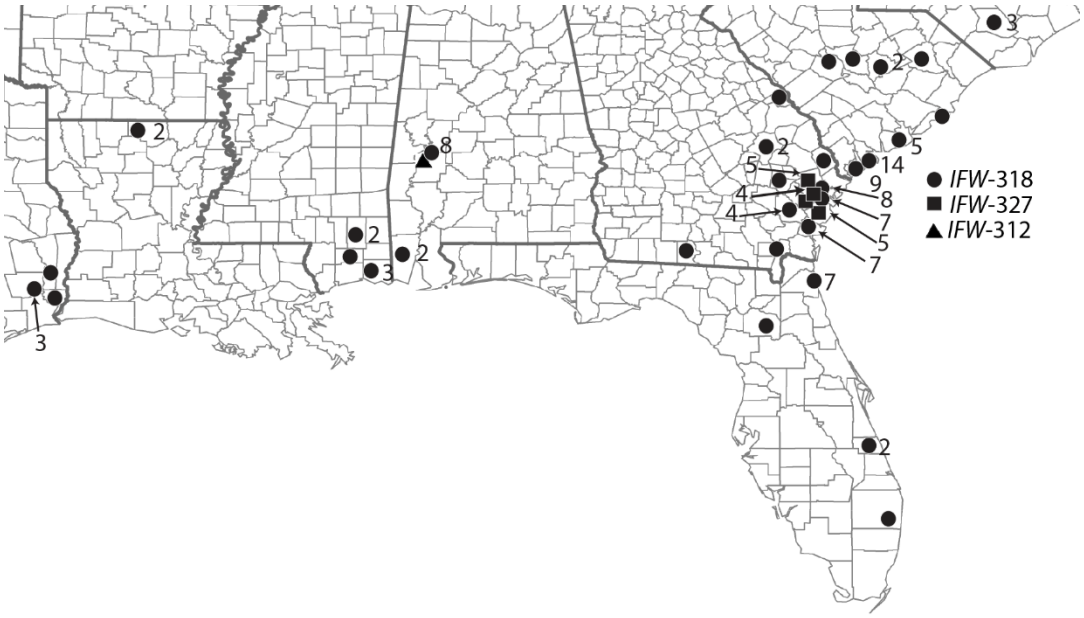

Figure 4

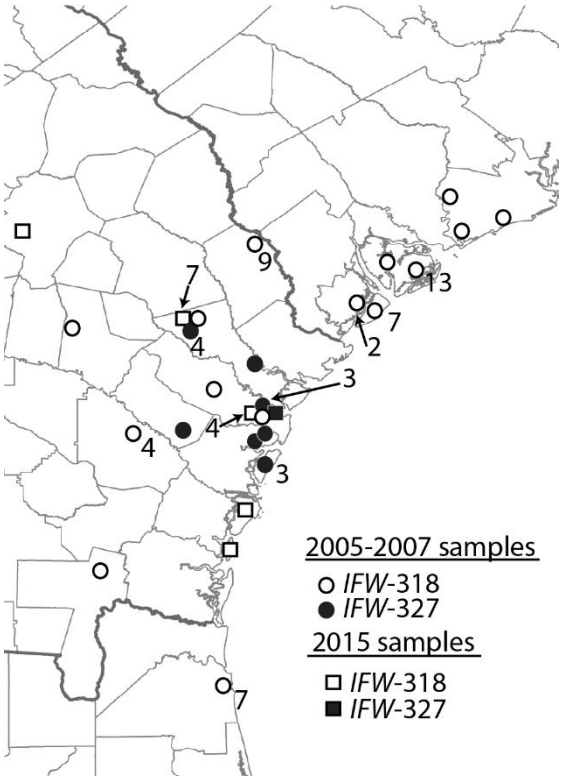
9
7
13
7
4
2
3
4
4
3
7
2005-2007 samples
IFW-318
IFW-327
2015 samples
IFW-318
IFW-327

Figure 5

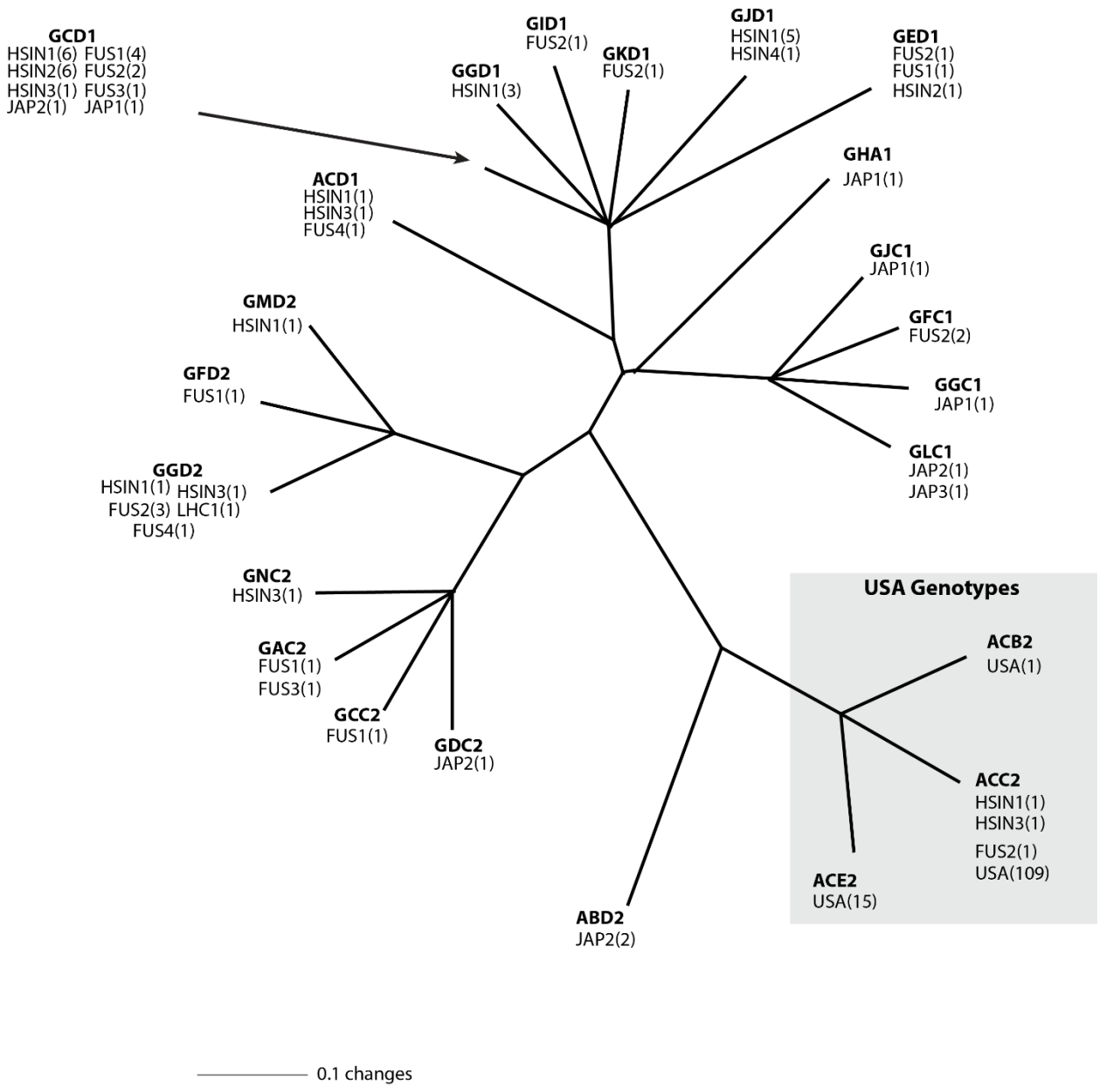
UPGMA
GCD1
HSIN1(6) FUS1(4)
HSIN2(6) FUS2(2)
HSIN3(1) FUS3(1)
JAP2(1) JAP1(1)
GID1
FUS2(1)
GKD1
FUS2(1)
GGD1
HSIN1(3)
GJD1
HSIN1(5)
HSIN4(1)
GED1
FUS2(1)
FUS1(1)
HSIN2(1)
GHA1
JAP1(1)
ACD1
HSIN1(1)
HSIN3(1)
FUS4(1)
GJC1
JAP1(1)
GMD2
HSIN1(1)
GFC1
FUS2(2)
GFD2
FUS1(1)
GGC1
JAP1(1)
GLC1
JAP2(1)
JAP3(1)
GGD2
HSIN1(1) HSIN3(1)
FUS2(3) LHC1(1)
FUS4(1)
GNC2
HSIN3(1)
USA Genotypes
ACB2
USA(1)
GAC2
FUS1(1)
FUS3(1)
GCC2
FUS1(1)
GDC2
JAP2(1)
ACC2
HSIN1(1)
HSIN3(1)
FUS2(1)
USA(109)
ACE2
USA(15)
ABD2
JAP2(2)
0.1 changes

Figure 6

CHAPTER 3. GENERAL CONCLUSIONS

Analysis of the genetic diversity in the USA population and Taiwan and Japan populations of *Raffaelea lauricola* has indicated that the USA population has undergone a severe genetic bottleneck and that the spread of the laurel wilt epidemic was caused by a single introduction of *R. lauricola*. High genetic diversity and two mating types were found in Taiwan and Japan, suggesting that cryptic sex may occur in native populations of *R. lauricola*. Two strains, identical in genotype aside from one genetic marker, were initially detected near Savannah, Georgia where *R. lauricola* and *Xyleborus glabratus* were likely introduced. Although the most common genotype of *R. lauricola* was found in Taiwan populations, the population genetics of *R. lauricola* in other Asian countries where *X. glabratus* is native must be investigated to determine the original source of the USA population.

Only one mating type (MAT2) of *R. lauricola* was detected in the USA. This study stresses the importance of preventing the introduction of new strains of *R. lauricola* to the USA, especially strains of the MAT1 mating type, in order to avoid genetic recombination of the fungus and the rise of a more aggressive strain of *R. lauricola*.

Recommendations for Future Research

This study raises questions about the mating type genes of *R. lauricola* and whether this fungal pathogen is truly capable of sexual reproduction. The partial sequences of the *MAT1-2-1* and *MAT1-1-3* mating type genes of *R. lauricola* could be used as a basis for obtaining complete sequences of the *MAT* locus in this species. Further attempts could be made to cross the two putative mating types in the laboratory to discover a sexual state in *R. lauricola*.

The *MAT* locus has yet to be studied in other *Raffaelea* spp., but the organization of the mating type genes and distributions in natural populations may provide insight into their mating systems, as it is likely that many *Raffaelea* spp. can form a sexual state in their life cycle. Obtaining sequences of the *MAT1-1* and *MAT1-2* idiomorphs would shed light on the evolutionary history and divergence of *Raffaelea* from *Ophiostoma* or *Leptographium*. If lineages within *Raffaelea* are truly asexual, then the presence of only one mating type might be expected in those lineages. Phylogenetic analysis of various representatives of *Raffaelea* spp. with the mating type genes as well as other genes may provide insight into the evolutionary history of *Raffaelea*, which has been little explored.

Disease management tactics for *R. lauricola* and other potential ambrosia fungal plant pathogens could be improved by expanding our knowledge of the genetics and mating type systems of *R. lauricola* and other *Raffaelea* spp. This research would also increase our understanding of the ambrosia beetle symbiosis.