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ORIGINAL PAPER

No rest for the laurels: symbiotic invaders cause unprecedented damage to southern USA forests

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Abstract Laurel wilt is an extraordinarily destructive exotic tree disease in the southeastern United States that involves new-encounter hosts in the Lauraceae, an introduced vector (*Xyleborus glabratus*) and pathogen symbiont (*Raffaelea lauricola*). USDA Forest Service Forest Inventory and Analysis data were used to estimate that over 300 million trees of redbay (*Persea borbonia* sensu lato) have succumbed to the disease since the early 2000s (ca 1/3 of the pre-invasion population). In addition, numerous native shrub and tree species in the family are susceptible and threatened in the Western Hemisphere. Genetic markers were used to test the hypothesis that the vector and pathogen entered North America as a single introduction. With a portion of the *cytochrome oxidase I* gene, a single *X. glabratus* haplotype was detected in the USA. Similarly, Amplified Fragment Length Polymorphisms indicated that 95% (54 of 57) of the isolates of *R. lauricola* that were examined were of a single clonal genotype; only minor variation was detected in three polymorphic

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isolates. Similar levels of disease developed after swamp bay (*P. palustris*) was inoculated with each of the four genotypes of *R. lauricola*. It is proposed that a single founding event is responsible for the laurel wilt epidemic in the United States.

Keywords Laurel wilt · Redbay ambrosia beetle · *Xyleborus glabratus* · *Raffaelea lauricola* · *Persea borbonia* · Invasive species · Forest disease · Forest Inventory and Analysis

Introduction

Ecosystems are undergoing unprecedented changes, driven in large part by global climate change and invasive non-native species (Anderson et al. 2004; Trumbore et al. 2015; Pautasso et al. 2015). In particular, forests are being decimated by exotic insects and microbes (Anderson et al. 2004; Smith et al. 2011; Boyd et al. 2013; Trumbore et al. 2015; Wingfield et al. 2016). For example, forest ecosystems in the northern hemisphere have been irreversibly altered by invading diseases, such as chestnut blight (*Cryphonectia parasitica* [Murr.] Barr) (Anagostakis 1987) and ash dieback (*Hymenoscyphus fraxineus* [T. Kowalski] Baral, queloz & Hosoya) (Pautasso et al. 2013), and insects, such as the emerald ash borer (*Agrilus planipenis* Fairmaire) (Herms and McCullough 2014) and hemlock woolly adelgid (*Adelges tsugae* Annand) (Vose et al. 2013).

Understanding the ecology of invasive species is challenged by uncertainties of how novel organismal assemblages will interact in new environments (Grosholz 2005; Simberloff 2006; Jackson 2015). Propagule pressure, invader genetic plasticity, host susceptibility and diversity, and many other biotic and abiotic factors interact to influence invader establishment and determine whether, or the extent to which, their range expands after introduction (Kolar and Lodge 2001; Lockwood et al. 2005; Drake and Lodge 2006).

In most animals, a single introduction of a few individuals is unlikely to result in establishment (Liebhold and Tobin 2008; Engering et al. 2013). Clonality, due to parthenogenesis, sib-mating and/or vegetative propagation, has been hypothesized to increase invasiveness in certain taxa, such as water fleas and aquatic plants (Roman and Darling 2007) because it eliminates the 'twofold cost of sex' and Allee effects such as inbreeding depression and mate-finding (Roman and Darling 2007; Fisher et al. 2012; Engering et al. 2013). For vectors of pathogens, especially insects that transmit fungal pathogens of trees, little is known about how their reproductive strategies and genetic diversity affect their ability to invade new ecosystems.

Laurel wilt is a tree disease caused by a nutritional fungal symbiont, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva, of the parthenogenic redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Fig. 1) (Fraedrich et al. 2008; Harrington et al. 2008). *Xyleborus glabratus* was first detected in North America in 2002 (Rabaglia et al. 2006). Although its introduction was initially considered unimportant, (ambrosia beetles rarely damage healthy trees) the subsequent epidemic of laurel wilt caused a re-examination of the importance of these insects in forest health (Hulcr and Dunn 2011; Ploetz et al. 2013). This disease has devastated mature trees of redbay, *Persea borbonia* L. Spreng., and other native *Persea* species, and has dramatically altered forest composition and dynamics throughout the southeastern United States (Fraedrich et al. 2008; Evans et al. 2013; Spiegel and Leege 2013; Cameron et al. 2015). In addition, several other species in the Lauraceae have been affected by laurel wilt, including avocado, *Persea americana* Mill. (Mayfield et al. 2008), and three critically endangered natives of the southeastern USA, *Lindera melissifolia* (Walter) Blume, *Litsea aestivalis* (L.) Fernald and *Licaria triandra* (Sw.) Kosterm., which face possible extinction due to the disease (Fraedrich et al. 2011; Ploetz and Konkol 2013).

Molecular data are used increasingly to examine the establishment and spread of invasive diseases and pests. Previously, the *cytochrome oxidase I (COI)* gene was used to characterize populations of diverse insects (Cognato et al. 2011a; Dole et al. 2010). Likewise, Amplified Fragment Length Polymorphism (AFLP) markers have been used to examine fungal plant pathogens. AFLPs enable the screening of many loci in a fungal genome without prior sequence knowledge or primer design (McDonald 1997; Meudt and Clarke 2007). In addition, simple sequence repeat (SSR) loci can be highly variable and phylogenetically informative (Selkoe and Toonen 2006).

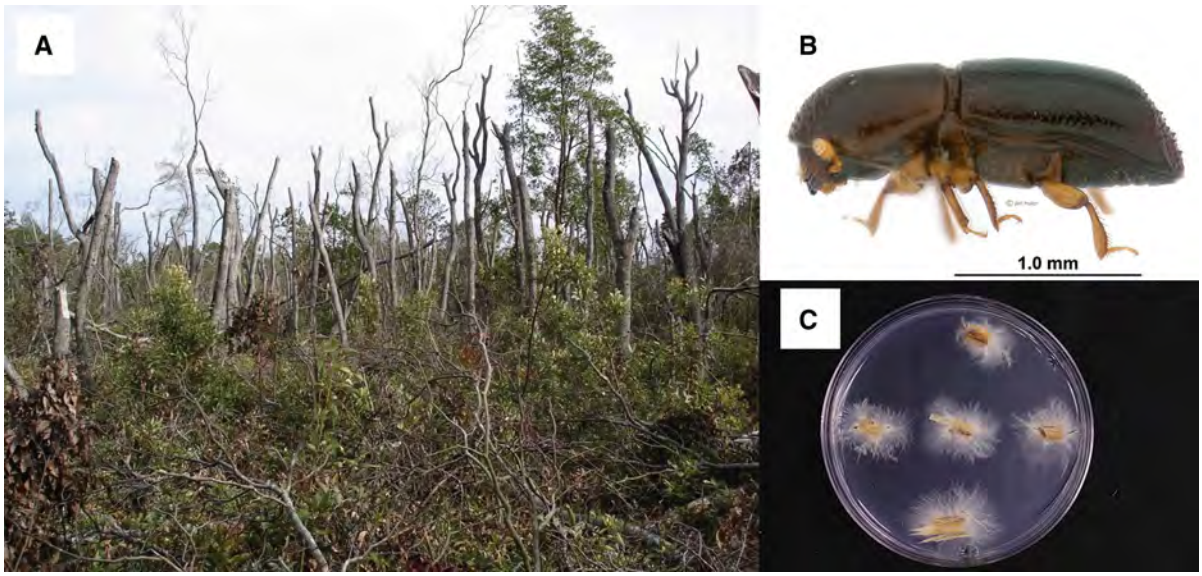

Fig. 1 Laurel wilt and its associates; **a** redbay (*Persea borbonia*) mortality due to laurel wilt, **b** the vector, female redbay ambrosia beetle (*Xyleborus glabratus*), **c** fungal pathogen (*Raffaelea lauricola*). Photos by: **a** Chip Bates, Georgia Forestry Commission. **b** Jiri Hulcr, University of Florida

The emergence of laurel wilt as a destructive force was examined in forests of the Atlantic Coastal Plain, a global biodiversity hotspot (Noss et al. 2015). With AFLPs and *COI* sequences, population structures were examined for, respectively, *R. lauricola* and *X. glabratus* in their introduced range. The genetic data were then used to infer invasion events in the southeastern United States. USDA Forest Service Forest Inventory and Analysis (FIA) data were used to estimate the total number of redbay trees that have been killed by laurel wilt since its arrival to the United States.

Materials and methods

Xyleborus glabratus sample preparation and analyses

From 2010 to 2012, 14 adult females of *X. glabratus* were collected from Florida (Bradford Co.), Georgia (Bryan, Glynn and Long Co.), Louisiana (Union Co.), Mississippi (Harrison and Jackson Co.) and South Carolina (Berkeley Co.) in Lindgren funnel traps baited with combinations of phoebe and manuka oil (Hanula and Sullivan 2008), and/or mesh bags filled with redbay foliage and twigs. Individuals were collected in infested stands and preserved in 95% (v/v) ethanol within 24 h of capture. No *X. glabratus* males were analyzed because they are wingless due to haplodiploidy, and were not captured in the funnel traps. DNA was extracted from preserved specimens and a 600 bp fragment of the 5′ end of the *COI* gene was amplified via PCR and directly sequenced using Sanger sequencing as described by Cognato et al. (2015). A consensus sequence was created for both DNA strands using Sequencer 5.3 (Ann Arbor, MI).

Raffaelea lauricola sample preparation and genetic analyses

In a separate experiment, a set of *R. lauricola* fungal isolates were collected from diverse hosts in South Carolina, Georgia, Florida and Mississippi (during the period from 2004 to 2010, representing the early stages of the laurel wilt epidemic) (Table S1); 36 were recovered by MAH, and 21 were provided by S. W. Fraedrich, (Southern Research Station, USDA Forest Service, Athens, GA). To ensure that each of the fungal samples tested were single-spore derived isolates, a growing subculture was streaked across CSMA culture media (Harrington 1981) with a sterile glass rod, and later a single growing conidium was excised via scalpel and transferred to fresh media for further analysis. A total of 57 single-spore isolates

were obtained for this study (Table S1). Conidia of each isolate were collected and macerated in acid-washed sand at room temperature before extracting DNA (Dreaden et al. 2014).

AFLP markers for the 57 isolates of *R. lauricola* were PCR amplified using fluorescently labeled primers as described by Kubisiak et al. (2011). Six primer pairs were tested against the panel of fungal isolates. All *Eco*RI selective primers possessed one selective nucleotide and all *Mse*I selective primers possessed two selective nucleotides (Table 1). The Interdisciplinary Center for Biotechnology Research Genetic Analysis Laboratory (University of Florida) provided fragment length analysis services, and samples were run on an ABI3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA) using GeneScan 600 LIZ size standard (Applied Biosystems Inc., Foster City, CA). Results were analyzed using GeneMarker V1.70 software (SoftGenetics LLC, State College, PA) with parameters set as: peak height threshold = 50, minimum fragment length = 50, smoothing enabled, and minimum peak score set to fail <1 check <1 pass. All polymorphic fragments were confirmed by repeating the AFLP procedure using DNA samples that had been extracted from independent cultures of each isolate. AFLP band presence/absence data were analyzed with POPGENE v1.31 to assess observed number of alleles, effective number of alleles, Nei's gene diversity and Shannon's Information index.

To further explore genetic relationships among four AFLP genotypes that were discovered above, SSR loci were screened. A sub-sample of three isolates with identical AFLP profiles (PL159, PL570, PL571) were selected as representatives of the predominant clonal genotype, and were compared to the three polymorphic AFLP fungal genotypes (PL388, PL692, PL735) (Table 1). In addition, flanking regions of the SSR loci were also sequenced (Rossetto et al. 2002; Germain-Aubrey et al. 2016). Primers were developed for all listed loci in Dreaden et al. (2014). Loci and primer sequences were previously noted for IFW and CHK in Dreaden et al. (2014), with four loci, 2IN, CPL, QI5, 7KC, described here (Table S2). Sequencing reactions were conducted at the Interdisciplinary Center for Biotechnology Research Genetic Analysis Laboratory at the University of Florida, Gainesville. Contigs for the forward and reverse sequences were created, aligned and edited with Geneious 5.4.6 software (Biomatters Limited, Auckland, New Zealand).

Raffaelea lauricola virulence assays and statistical analysis

The pathogenicity and virulence of isolates of *R. lauricola* were assessed. Isolates PL571 (represented the predominant AFLP profile), PL388, PL692 and PL735 (the three polymorphic isolates) were tested. Swamp bay (*P. palustris*) plants with a single dominant stem were grown in 12.5 L containers until approximately 1.5 m tall. Plants were grown in a greenhouse with supplemental lighting (16:8 diurnal light) and temperature was maintained at 21 °C day/18 °C night with an evaporative cooler pad and fan system. Four plants were inoculated with each isolate (1.05×10^5 conidia per plant) or water (mock-inoculated control), as described previously (Hughes et al. 2015b). The plants

Table 1 Assessment of *Raffaelea lauricola* genetic diversity via Amplified Fragment Length Polymorphism (AFLP) analysis

Primer pairs	*Eco*RI primer extension	*Mse*I primer extension	Fragments scored	Fragment size range (bp)	Polymorphic isolates
E9/M9	A	CG	40	36–520	PL388, PL735
E12/M9	G	CG	34	44–524	–
E11/M20	C	CA	54	32–570	–
E11/M21	C	CT	34	34–576	PL692
E12/M20	G	CA	40	31–541	–
E12/M21	G	CT	16	34–332	PL388

Total fragments scored per fungal isolate = 218. Mean DNA fragments per primer pair = 36. Descriptive statistics generated by POPGENE v1.31 are as follows: observed number of alleles = 1.0183 ± 0.1345, effective number of alleles = 1.0007 ± 0.0048, Nei's gene diversity = 0.0006 ± 0.0046 and Shannon's Information Index = 0.0016 ± 0.0119

were arranged in a randomized complete block design and the experiment was conducted twice.

Disease was assessed every fourth day according to an interval scale where 0 = asymptomatic, 1 = 1–10%, 2 = 11–20%; … 10 = 90–100% of the canopy with wilt (Hughes et al. 2015b). At 52 days post-inoculation, a 10 cm portion of the main stem at 20 cm above ground level was removed and de-barked, and the percentage of internal tissue discoloration around the circumference was visually estimated using the above scale. Statistical analyses were conducted as in Hughes et al. (2015b). Briefly, internal and external disease severity scores were converted to proportions of the mid-point of scale ranges (i.e., 50–60% = 0.55). The area under the disease progress curve (AUDPC) was calculated using the mid-point rule (Campbell and Madden 1990). The rate of disease development was estimated by the NLIN procedure in SAS (SAS Institute, Cary, NC) using the Gompertz nonlinear model, which was chosen due to goodness of fit, residual distributions and regression coefficient of determination (R^2) (Hughes et al. 2015b). Analysis of variance was performed using the GLM procedure, followed by Tukey's multiple comparisons of least-squared means at $\alpha = 0.05$. Because no significant differences were detected between block, experiment and their interactions at $\alpha = 0.05$, the data from both experiments were combined (data not shown, n = 32).

Range estimates for the total number of redbay trees killed

Data from the USDA Forest Service Forest Inventory and Analysis (FIA) program were used to estimate the number of redbay trees killed by laurel wilt across the southeastern USA. The FIA program manages an annualized system of field survey plots that provides consistent sampling of the nation's forest lands (McRoberts 2005). Under this system, a spatially distributed subset of the FIA plots in a state are measured each year (Shaw et al. 2005). In the southeastern USA, a full inventory cycle combining these subsets is typically completed over a period of five or seven years, depending on the state. Details regarding the FIA plot design and measured attributes are provided elsewhere (Bechtold and Scott 2005). To enable broad-scale geographic analyses, plot-level measurements that are initially calculated on a per-unit-area basis (e.g., tree density per hectare) are translated into corresponding estimates for larger geographic units (e.g., the number of trees in a county) based on expansion factors developed by the FIA program, which indicate the exact amount of area that each plot represents (Hicke et al. 2007). Generally, a plot represents about 2400 forested hectares (Shaw et al. 2005).

For this analysis, trees ≥2.54 cm diameter at breast height (dbh) were labeled according to their species identifier within the FIA database (i.e., redbay-*Persea borbonia)*; however, due to the lack of identifiers for the closely related swamp bay (*P. palustris* [Raf.] Sarg.) and silk bay (*P. humilis* Nash), it is likely that these species were included as a species complex (*P. borbonia* sensu lato). Two sets of FIA plots, spanning multiple years, were selected for each state where redbay is found (Table 2). The first set of plots was selected from the period before laurel wilt was detected in a state (i.e., the "pre-invasion inventory period"), while the second set was selected from the period after detection (i.e., the "current inventory period"), with a few exceptions. For Virginia, where laurel wilt has not yet been found, and Texas, where the disease was only detected in 2015, the current set came from the most recently completed FIA inventory cycle, while the pre-invasion set was from the inventory cycle prior to that. The same approach was adopted for Louisiana, where laurel wilt was first detected in 2014, but on an alternate host, sassafras (*Sassafras albidum* [Nutt.] Nees), and is yet to be reported on redbay. Whenever possible, full inventory cycles were used when performing calculations.

The analysis focused on current-inventory FIA plots. For a given state, the numbers of live and dead redbay trees found on individual plots were converted into per-hectare rate estimates. The mean live and dead per-hectare estimates across these plots were then multiplied by the state's total forest area (in hectares) to estimate the total number of live and dead redbay trees in the current inventory period. The set of current-inventory plots in each state was also compared with the pre-invasion set to identify redbay trees that represented new growth since the pre-invasion period.

Whether a dead redbay tree on an FIA plot was killed by laurel wilt was determined using an FIA attribute field ("agentcd") specifying cause of death in several categories: insect, disease, fire, animal, weather, vegetation (e.g., competition, suppression),

Table 2 Estimated redbay (*Persea borbonia*) mortality from laurel wilt

State	Pre-invasion inventory period		Laurel wilt first reported	Current inventory period				
	Inventory period	Live trees × 1 M		Inventory period	Live trees including regeneration × 1 M[c]	Dead trees × 1 M[d]	Laurel wilt-killed redbay × 1 M[d]	Percent laurel wilt mortality[e]
Alabama	2001–2005	23.8 (6.6)	2011	2011–2015	41.7 (9.4)	22.1 (6.9)	0.0 (0)	0.0%
Florida	2002–2004	92.2 (9.8)	2005	2009–2014	134.6 (12.8)	96.5 (11.6)	68.9 (9.0)	36.5%
Georgia	1999, 2001–2004	56.5 (8.0)	2004	2008–2014	101.5 (11.7)	157.5 (20.2)	144.4 (18.8)	67.5%
Louisiana[a]	2001–2005, 2008	3.9 (1.6)	2014	2009–2014	11.8 (3.6)	9.7 (3.7)	0.0 (0)	0.0%
Mississippi[b]	2006[b]	47.0 (8.6)	2009	2009–2015	62.1 (10.5)	7.0 (2.1)	1.1 (0.5)	2.1%
North Carolina	2003–2007	160.4 (19.7)	2011	2011–2015	254.9 (25.4)	144.8 (17.5)	30.0 (4.9)	9.8%
South Carolina	2002–2004	70.6 (9.1)	2004	2008–2015	105.7 (11.2)	102.2 (12.0)	71.8 (8.7)	41.6%
Texas[a]	2004–2008	30.3 (6.0)	2015	2009–2014	45.9 (9.0)	19.3 (3.8)	0.0 (0)	0.0%
Virginia[a]	2002–2007	11.5 (3.7)	n/a	2009–2014	21.5 (8.1)	1.6 (0.8)	0.0 (0)	0.0%
All states		496.3 (28.3)			779.7 (37.8)	560.7 (32.8)	316.3 (23.1)	29.9%

Standard deviations of the estimates are shown in parentheses

[a] See Materials and methods for explanation of the pre-invasion and current inventory cycles in Louisiana, Texas, and Virginia

[b] Mississippi completed a "close-out" periodic inventory in 2006, equivalent to a full (i.e., multi-year) inventory cycle under the annualized system

[c] Totals include new growth since the pre-invasion inventory period

[d] Dead tree estimates only include trees that were alive prior to pre-invasion inventory period

[e] Calculated as a percentage of all redbay trees, live and dead, that were present prior to invasion, i.e., excluding subsequent new growth

silviculture or land clearing activity, or uncertain (i.e., no specified cause of death). Any dead redbay tree in the current inventory period for which the recorded cause of death was insect or disease was assumed to have been killed by laurel wilt, based on the lack of other insects and diseases associated with redbay mortality. Additionally, a proportion of the trees labeled as killed by fire, silviculture, or land clearing activity—or where the cause of death was uncertain—were assumed to have actually been killed by laurel wilt; these large-scale (i.e., forest stand or larger) disturbances may have obscured the presence of laurel wilt on redbay trees in the affected areas. This proportion was estimated at the state level by setting aside trees killed by fire, silviculture, land clearing, or unknown causes, and calculating the fraction of all remaining dead trees that were killed by insect or disease. For each state, this proportion was then multiplied by the number of set-aside trees, and the product was added to the number of trees killed by insect or disease, yielding an estimate of the total number of trees in each state that were killed by laurel wilt. Standard deviations of the estimates were calculated according to methods described in Scott et al. (2005). Percent laurel wilt mortality in each state was calculated as a percentage of all redbay trees, live or dead, that were present prior to invasion, i.e., excluding new growth.

Maps of percent laurel wilt mortality and wilt-killed trees per hectare were generated via ordinary kriging of mortality measurements on FIA plots containing redbay. Only plots that were measured in both the pre-invasion and current inventory periods ($N = 617$) were included in the analysis. Trees that were not present in the pre-invasion period (i.e., new growth) were omitted from calculations. Percent laurel wilt mortality values for each plot were subjected to an arcsine transformation prior to interpolation. For both variables, a second-order polynomial trend was removed and kriging was performed on the residuals using a spherical semivariogram model. Interpolation was based on a circular neighborhood, and interpolated values were calculated using a minimum of 10 and maximum of 20 neighbors. Non-forest pixels were omitted from both resulting maps, as were pixels with zero probability of redbay presence based on indicator kriging of all FIA plots in the redbay range.

Results

Analysis of partial *cytochrome oxidase I* sequences of *X. glabratus*

A single *COI* haplotype of *X. glabratus* was evident among the 14 assayed specimens, which was previously reported for a specimen collected in 2005 from GA (Cognato et al. 2011a, Genbank # HM064127).

Amplified Fragment Length Polymorphism (AFLP) analysis of *R. lauricola*

A total of 218 fragments were generated with the six AFLP primer pairs, with a range of 16–54 fragments per primer pair (Table 1). To determine the reproducibility of fragment amplification, duplicate preselective reactions were prepared for ten restriction-ligation reactions and amplified. In addition, duplicate DNA samples were prepared for 28 isolates using independent cultures, and AFLP templates prepared from these biological replicates were also amplified using the same primer pairs. Identical results were obtained for all technical and biological replicates (data not shown), indicating that the AFLP procedure was reliable and reproducible.

Only four of the 218 loci that where scored were polymorphic (1.8%). Of the 57 isolates examined, 54 had identical AFLP profiles. Among the three that differed, isolate PL735 lacked a 179 bp fragment amplified with primers *Eco*RI-A/*Mse*I-CG; isolate PL692 produced an extra 494 bp fragment with the *Eco*RI-C/*Mse*I-CT primers; and isolate PL388 lacked a fragment of 125 bp when amplified with the *Eco*RI-A/*Mse*I-CG primers and lacked a 330 bp when amplified with *Eco*RI-G/*Mse*I-CT primers (Table 1). In total, the observed number of alleles = 1.0183 ± 0.1345, effective number of alleles = 1.0007 ± 0.0048, Nei's gene diversity = 0.0006 ± 0.0046 and Shannon's Information Index = 0.0016 ± 0.0119 (POPGENE v1.31). The four polymorphic fragments were confirmed with DNA from independent cultures of each isolate. All three polymorphic isolates were recovered from redbay. PL388 and -692 were recovered from adjacent counties in Georgia (Pierce and Appling Co., respectively) and PL735 from Columbia Co. Florida (Fig. 2).

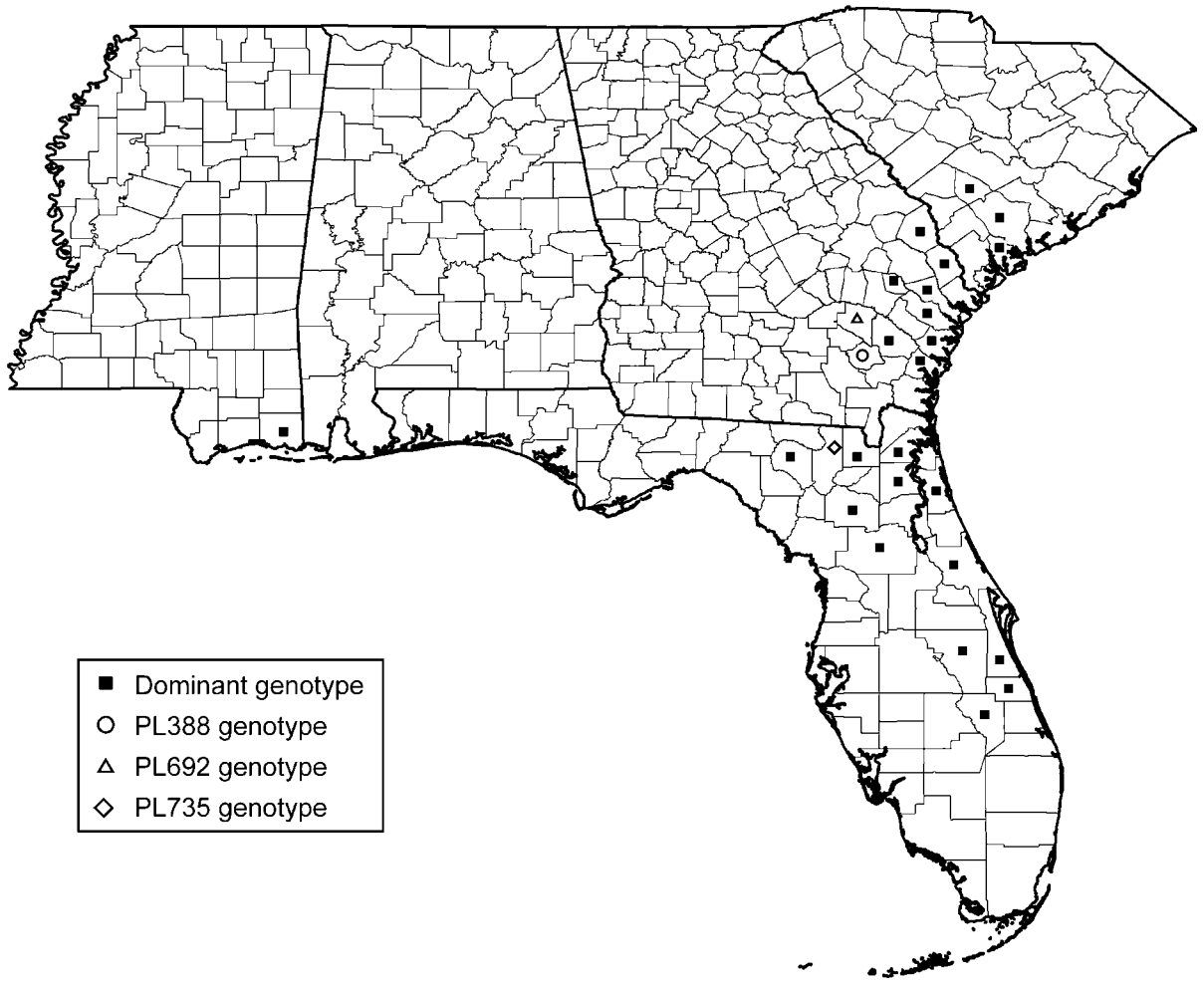

Fig. 2 Counties where *Raffaelea lauricola* isolates were collected from 2005 to 2010 for AFLP study. Isolate genotypes are denoted by *symbols*

Analysis of *R. lauricola* simple sequence repeat (SSR) and flanking regions

Microsatellite loci and flanking region sequences revealed 100% nucleotide identity among the six isolates at six loci. The three isolates containing polymorphic AFLP profiles (PL388, PL692, PL735) were identical to those with the clonal dominant AFLP profile (PL159, PL570, PL571). Total amplicon sizes ranged from 37 to 210 bp, with di and tri-nucleotide repeats ranging from 12 to 33 bp. Flanking regions were 19–177 bp in length (data not shown).

Pathogenicity and virulence assays of *R. lauricola*

All plants inoculated with *R. lauricola* isolates displayed characteristic laurel wilt symptoms within 21 days of inoculation, and water-inoculated controls remained asymptomatic. This confirms the pathogenicity of the isolates tested. Mean external disease severity ratings were similar (94–95%, $P = 0.84$) and all inoculated trees eventually died regardless of the isolate that was used. No significant differences were detected among the isolates tested in mean rate of disease development (0.15–0.16, $P = 0.67$), mean internal disease severity (vascular discoloration) (83–85%, $P = 0.95$) and mean area under the disease progress curve (AUDPC) (19.0–20.6, $P = 0.69$) (data not shown). Thus, the minor polymorphisms in AFLP profiles had no observable relation to disease development on swamp bay. *R. lauricola* was recovered from all inoculated plants, but not from water-inoculated controls.

Range estimates for the total number of redbay trees killed

We used FIA plot data to calculate the abundance of live and dead redbay in natural forests and how much of the observed redbay mortality was caused by laurel wilt. Of the approximately 1.1 billion redbay trees that were present across the study region prior to 2003, we estimate that nearly 320 million (~30%) have succumbed to laurel wilt (Fig. 3; Table 2). Most of the mortality has occurred in South Carolina, Florida, and especially Georgia, which has lost more than two-thirds of its pre-invasion redbay population due to laurel wilt. Overall, the regional redbay mortality pattern conforms to expectations based on the documented spread of laurel wilt since introduction (Table 2). Percent mortality is extremely high in Georgia, where *X. glabratus* was first detected in 2002, followed by South Carolina and Florida, where laurel wilt impacts were reported in 2004 and 2005, respectively (Fig. 3). North Carolina, where the disease was discovered in 2011, has experienced moderate wilt-related redbay losses in the southern portion of the state (Fig. 3b). However, the mortality is comparatively low in percent terms (Fig. 3a; Table 2) because of the state's very large redbay population. Unlike Louisiana and Texas, where laurel wilt was only discovered recently, the low level of observed laurel wilt mortality in Mississippi and the lack of wilt-caused mortality in Alabama defy expectations given when the disease was reported in each state (2009 and 2011, respectively). In both states, actual redbay mortality due to laurel wilt may be underestimated because tree death from other causes such as fire and silvicultural activity, which are often determined at the forest stand level, could hide the presence and impact of the disease on individual trees. Indeed, fire and silviculture are so extensive throughout the study region that wilt-caused mortality could also be underestimated elsewhere, so the estimates presented here should be considered as conservative. Furthermore, we estimate that regeneration has added just over 280 million redbay trees to the region-wide population since invasion (Table 2), which is not enough to offset the mortality caused by the disease. Moreover, once beyond the sapling stage, most of this new growth will become suitable for *X. glabratus* attack and succumb to laurel wilt (Cameron et al. 2015).

Discussion

Laurel wilt has killed at least 320 million redbay trees, nearly 1/3 of the pre-invasion population. Clearly, additional individuals of this species and other members of the Lauraceae will be killed in the future. Although this study focused on a single host species, redbay (*P. borbonia*), other suscepts have undoubtedly also suffered significant population losses to this disease. Thus far, 14 species of Lauraceae are known to be susceptible to *R. lauricola* (Hughes et al. 2015a). They range across the continental USA and Europe, and occupy diverse ecosystems. Shearman et al. (2015) assessed the population dynamics of live redbay trees (>2.5 cm diameter) using FIA data and suggested the possible start of a range-wide population decline due to laurel wilt in the USA. Although laurel wilt-killed redbays produce many stems from basal sprouts and seedling regeneration can be abundant, this subsequent regrowth is not sufficient to offset the mortality caused by laurel wilt, especially since much of this regeneration will later succumb to the disease (Cameron et al. 2015). Additional impacts of the epidemic may include the decline of associated fauna and flora such as the Palamedes swallowtail, *Papilio palamedes* (Drury), an obligate redbay herbivore (Chupp and Battaglia 2014), and the orange-fringed orchid, *Platanthera ciliaris* (L.) Lindl., which depends on *P. palamedes* for pollination (Robertson and Wyatt 1990; Chupp et al. 2015). Extensive mortality of swamp bay (*P. palustris*) in tree islands of the Florida Everglades could tip the balance in favor of invasive plant species and ultimately cause changes in hydrologic patterns (Rodgers et al. 2014). Unknown multitrophic impacts may occur in Mexico and tropical Central and South America, where a large suite of potential Lauraceae hosts exists. Finally, economic effects in the commercial avocado production region of south Florida (Mosquera et al. 2015; Ploetz et al. 2017a) will continue to worsen as the disease spreads. Time will tell when and whether the most important avocado-producing state, California, and world's most important producer, Mexico, will be affected.

The presence of a single *X. glabratus* haplotype suggests genetic homogeneity in the US population. In large populations, the over-production of females (as in haplodiploid ambrosia beetles) is likely to fix mitochondrial nucleotide mutations among populations, giving rise to many *COI* haplotypes. This

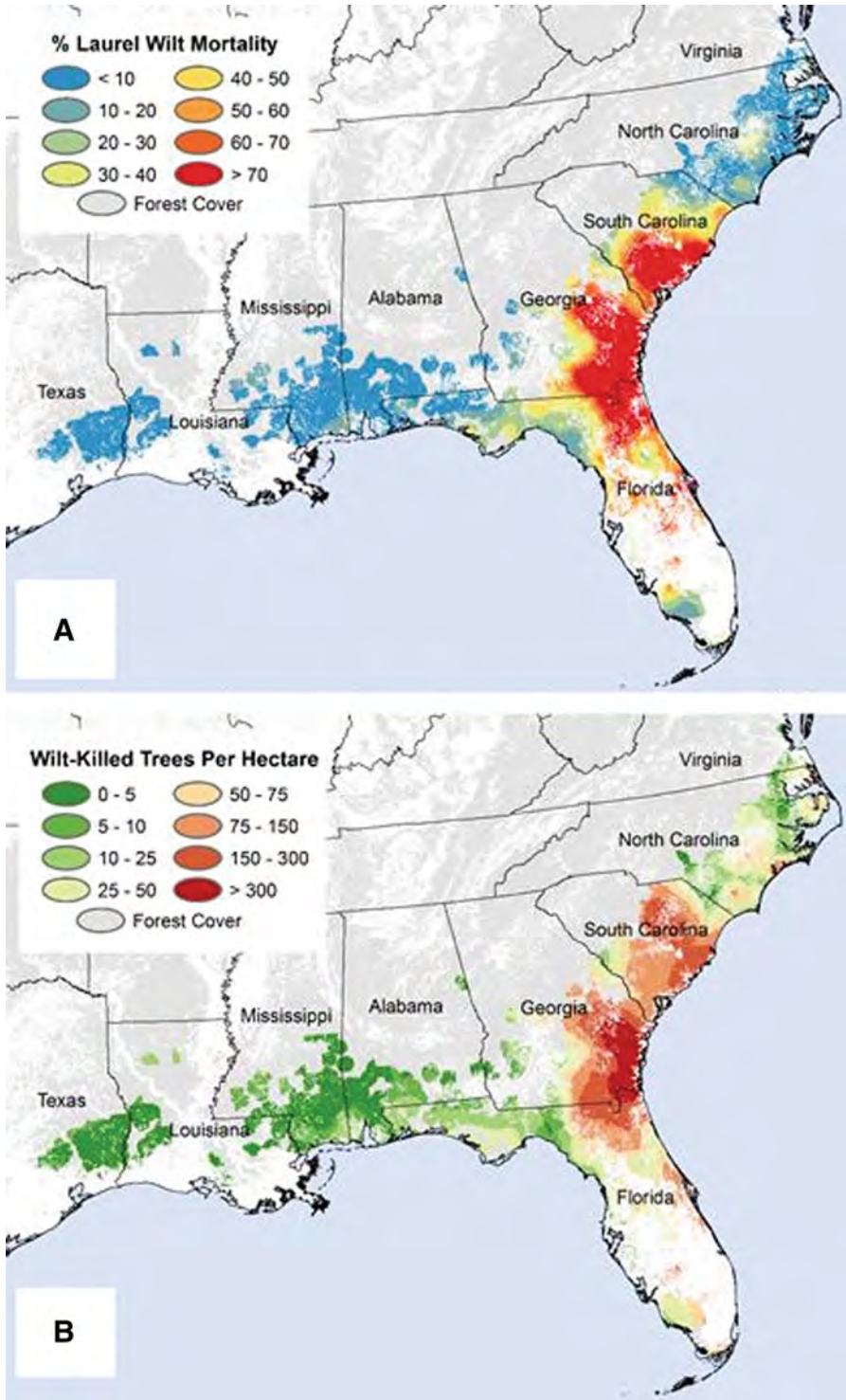

Fig. 3 Redbay (*Persea borbonia*) mortality caused by laurel wilt, based on spatial interpolation of Forest Inventory and Analysis plot data: **a** percent of laurel wilt mortality, **b** wilt-killed trees per hectare. Background layer depicting regional forest cover (in *gray*) developed from Moderate Resolution Imaging Spectroradiometer (MODIS) data by the USDA Forest Service Geospatial Technology and Applications Center

phenomenon of multiple unique haplotypes is observed in populations of Xyleborine ambrosia beetles within their native ranges (Dole et al. 2010; Cognato et al. 2015), and allows for the estimation of the number of likely introduction events, as well as the geographic origin of the invasion (Cognato et al.

2015). Similar to *X. glabratus*, *COI* sequence analyses also suggested single introductions of the *Xylosandrus amputatus* (Blandford) and *Euwallacea validus* (Eichhoff) ambrosia beetles into the US (Cognato et al. 2011b, 2015). Xyleborine ambrosia beetles, including *X. glabratus*, are extreme inbreeders (Kirkendal et al. 2015), in which females produce a few dwarfed and flightless haploid males that rarely leave their brood chamber, where they mate with their diploid sisters. These females disperse to start new families. Thus, one female has great invasive potential. In fact, given the haplodiploid and sib-mating population biology of *X. glabratus*, wherein female beetles can produce males asexually and females sexually to establish viable colonies, it is possible that the current epidemic in the USA originated from the introduction of a single female beetle. Analysis of more individuals of *X. glabratus* from the entire US range may detect variation not found in our analysis, and additional sampling in the native range of *X. glabratus* in Asia (Rabaglia et al. 2006) could help identify the source population for the US epidemic.

Raffaelea lauricola is the predominant fungal symbiont of *X. glabratus* in the USA, and although other *Raffaelea* species are present within the mycangia of the beetle (Harrington and Fraedrich 2010; Carrillo et al. 2014; Campbell et al. 2017), only *R. lauricola* is pathogenic (Dreaden et al. 2017). Harrington et al. (2011) recovered *R. lauricola* from the mycangia of *X. glabratus* in Taiwan and Japan, supporting the hypothesis that the fungus entered the USA with *X. glabratus* from Asia. Although the beetle is endemic in Asian forests and associated with native Lauraceae (Hulcr and Lou 2013), laurel wilt has not yet been reported in native forests. Outside of the US, laurel wilt has only been detected in Burma (Myanmar) in cultivated avocado (a non-native plant) (Ploetz et al. 2016). The absence of laurel wilt in Asian Lauraceae is hypothesized to result from co-evolution with *R. lauricola*, leading to selection for resistance in these host taxa (Ploetz et al. 2017b). The response of the Asian camphortree (*Cinnamomum camphora* [L.] J. Presl), now common in the southeastern USA, supports this hypothesis, as it usually develops only a mild branch dieback when infected with *R. lauricola* (Smith et al. 2009; Fraedrich et al. 2015).

The isolates of *R. lauricola* from the southeastern USA that were examined in the present study were remarkably uniform. Identical AFLP profiles were found in 95% (54 of 57) of the *R. lauricola* isolates that were assayed, and the differences that were evident in the polymorphic isolates were minor ($\approx$99% similarity to the dominant clonal genotype). We believe that these AFLP results are sufficient to show genetic homogeneity as these loci are throughout the genome and have resolved the population and phylogenetic relationships of several forest pathogens (Ivors et al. 2004; Kim et al. 2006; Duran et al. 2010). Moreover, to further compare the variation among the AFLP polymorphic isolates to the dominant single clonal genotype, six microsatellite loci were screened. Because no variation was detected in the dominant clone amongst the 218 AFLP fragments screened per isolate, a subset of three isolates were chosen to represent this genotype. When six SSR loci and their flanking sequences were compared, the three divergent isolates were identical to the dominant clone. No pathogenic variability was observed for isolates with different AFLP profiles.

Recently, Wuest et al. (2017) utilized diagnostic microsatellite markers, which were developed by Dreaden et al. (2014), as well as rDNA and mating-type loci to examine variation in US populations of *R. lauricola*. They also reported uniformity in the US population, and suggested a single introduction event. All their US isolates contained the same MAT 1-2-1 mating type gene allele for the 28S region and CHK SSR locus (Wuest et al. 2017). A small group of isolates from coastal Georgia collected early in the epidemic and a single isolate from Alabama had different alleles in IFW SSR locus. However, when this region was re-sampled these alleles were rare, leading the authors to suggest a simultaneous introduction of two *R. lauricola* genotypes, with the fitter of the two surviving and spreading the epidemic (Wuest et al. 2017). In the present study, there were no shared differences or unifying pattern among the three AFLP polymorphic isolates, suggesting that the observed variation was due to random mutation among restriction or primer-annealing sites. The genetic homogeneity of *X. glabratus* and *R. lauricola* samples collected from early in the laurel wilt invasion (2005–2010) represent clonal populations, which suggests a shared single introduction to the USA.

When compared to the US population, more variation was apparent in *R. lauricola* from Asia. Harrington et al. (2011) reported a single base pair substitution in the 28S rDNA of *R. lauricola* collected

from Taiwan and Japan when compared to the monomorphic US population. Dreaden et al. (2014) reported 14 additional TCT repeats at the IFW locus from a Japanese isolate. Wuest et al. (2017) reported 21 genotypes among 65 Asian isolates that they sampled. They noted MAT1 or MAT2 mating types in at nearly a 1:1 ratio, which they suggested might enable sexual reproduction of *R. lauricola* in Asia.

Other epiphytotics have been associated with clonal pathogens in new geographic regions. In Chile, a clonal population of *Phytophthora pinifolia* Alv. Durán, Gryzenh. & M.J. Wingf. is responsible for a new disease of *Pinus radiata* D. Don. called "daño foliar del pino" (Duran et al. 2010). Three distinct clonal lineages (NA1, NA2 and EU1) of the sudden oak death pathogen, *Phytophthora ramorum* Werres, De Cock & Man in 't Veld, have established in California, Washington/Canada and Europe, respectively (Grünwald et al. 2012). Even in long-established exotic tree diseases, such as chestnut blight and Dutch Elm disease, where genetic diversity can be high in established populations, the colonization of new regions by single clonal genotypes still occurs (Hoegger et al. 2000; Brasier 2001).

A single introduction, low initial propagule pressure and clonal populations are often considered negative factors in invader establishment (Lockwood et al. 2005; Drake and Lodge 2006; Engering et al. 2013). However, a conducive environment can overshadow many of these limiting factors and increase the chances of successful invasion (Drake and Lodge 2006; Duncan 2016). Establishment by many non-native ambrosia beetles typically occurs in the wetter and warmer areas of the USA, where climatic conditions support reproduction and development of the beetle and their fungal symbionts (Rassati et al. 2016). Climate-matching models indicated that *Xyleborus glabratus*, a native to tropical and subtropical Asia (Rabaglia et al. 2006), is well suited to the southeastern USA (i.e., the geographic range of redbay) (Koch and Smith 2008). However, its cold tolerance and impact on a temperate host, sassafras (*Sassafras albidum* [Nutt.] Nees), suggest that it could continue to spread to areas that lack redbay (Bates et al. 2013; Formby et al. 2013). We hypothesize that a favorable climate, abundance of susceptible hosts in the Lauraceae, haplodiploidy in *X. glabratus*, and the virulence of *R. lauricola* (Hughes et al. 2015b) enabled the laurel wilt pathosystem to establish itself in the USA, despite the challenges that otherwise would confront a single introduction of a clonal vector and pathogen.

Predicting future threats to forests has been identified as a key element of proactive natural resource management. In some cases, this can be accomplished by recognizing potential threats from invasive species in their native ranges. However, laurel wilt was a completely unexpected "Black Swan" event (Ploetz et al. 2013), as the disease has yet to be identified in natural hosts in Asia where the fungus and its vector originated. Laurel wilt's new status as a lethal threat in American forests should serve as a warning that unpredictable consequences can be associated with biological invasions. The ecological impacts of laurel wilt are arguably among the most spectacular ever recorded for a clonal vector and pathogen, and is the only example we are aware of that involves a symbiotic pair of organisms.

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