

Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt pathogen, *Raffaelea lauricola*, originated in Asia

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Abstract: The laurel wilt pathogen *Raffaelea lauricola* was hypothesized to have been introduced to the southeastern USA in the mycangium of the redbay ambrosia beetle, *Xyleborus glabratus*, which is native to Asia. To test this hypothesis adult *X. glabratus* were trapped in Taiwan and on Kyushu Island, Japan, in 2009, and dead beetles were sent to USA for isolation of fungal symbionts. Individual *X. glabratus* were macerated in glass tissue grinders, and the slurry was serially diluted and plated onto malt agar medium amended with cycloheximide, a medium semiselective for *Ophiostoma* species and their anamorphs, including members of *Raffaelea*. *R. lauricola* was isolated from 56 of 85 beetles in Taiwan and 10 of 16 beetles in Japan at up to an estimated 10 000 CFUs per beetle. The next most commonly isolated species was *R. ellipticospora*, which also has been recovered from *X. glabratus* trapped in the USA, as were two other fungi isolated from beetles in Taiwan, *R. fusca* and *R. subfusca*. Three unidentified *Raffaelea* spp. and three unidentified *Ophiostoma* spp. were isolated rarely from *X. glabratus* collected in Taiwan. Isolations from beetles similarly trapped in Georgia, USA, yielded *R. lauricola* and *R. ellipticospora* in numbers similar to those from beetles trapped in Taiwan and Japan. The results support the hypothesis that *R.*

lauricola was introduced into the USA in mycangia of *X. glabratus* shipped to USA in solid wood packing material from Asia. However differences in the mycangial mycoflora of *X. glabratus* in Taiwan, Japan and USA suggest that the *X. glabratus* population established in USA originated in another part of Asia.

Key words: *Cinnamomum* spp., Curculionidae, Lauraceae, mycangia, *Ophiostoma* spp., *Persea* spp., *Raffaelea ellipticospora*, *R. fusca*, *R. subfusca*, Scolytinae, vector

INTRODUCTION

Laurel wilt is a newly recognized disease on redbay (*Persea borbonia* (L.) Spreng.), swampbay (*P. palustris* (Raf.) Sarg.) and other members of the Lauraceae, including *Sassafras albidum* (Nutt.) Nees and commercial avocado (*Persea americana* Mill.), in the southeastern USA (Fraedrich et al. 2008). This lethal vascular wilt disease is caused by an ambrosia beetle symbiont, *Raffaelea lauricola* T.C. Harr., Aghayeva, & Fraedrich (Harrington et al. 2008). Species of *Raffaelea* Arx & Hennebert are asexual symbionts of ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) (Harrington et al. 2010), and the primary vector of *R. lauricola* is the exotic redbay ambrosia beetle, *Xyleborus glabratus* Eichh. (Scolytinae), which was introduced accidentally from Asia and first detected in 2002 at a port near Savannah, Georgia (Fraedrich et al. 2008, Harrington and Fraedrich 2010, Rabaglia et al. 2006). Extensive mortality of redbay was noted shortly after the appearance of the beetle, and *R. lauricola* was shown to be the cause (Fraedrich et al. 2008). The redbay ambrosia beetle is native to Asia (India, Myanmar, Japan, Taiwan), where it infests aromatic tree species, especially those in the family Lauraceae (Wood and Bright 1992). It is likely that the beetle arrived in USA in solid wood packing material, and it was assumed that the beetle brought *R. lauricola* with it (Fraedrich et al. 2008, Harrington et al. 2008, Harrington and Fraedrich 2010).

Adult ambrosia beetles generally bore galleries and lay eggs in the nutrient-poor sapwood of dead or dying trees (Farrell et al. 2001). Fungal symbionts grow in the sapwood and produce conidia from tightly packed conidiophores (sporodochia) and sprout cells, on which the adults and larvae feed (Batra 1963, 1967; Francke-Grosmann 1967; Harring-

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ton 2005). The fungi usually are carried tree-to-tree by the adults in specialized pouches called mycangia, in which the fungi multiply in a yeast phase nourished by secretions from surrounding gland cells (Batra 1963, Beaver 1989, Francke-Grosmann 1967). Like many other species of *Xyleborus*, *X. glabratus* has paired sacs (mycangia) near its mandibles for carrying fungal spores (Fraedrich et al. 2008). The mycangia of *X. glabratus* are tightly packed with fungal spores, and up to 30 000 colony-forming units (CFUs) of *R. lauricola* were estimated from surface-sterilized beetles collected in Georgia, USA (Harrington and Fraedrich 2010). *R. lauricola* was the most common of six species of the genus *Raffaelea* that were isolated from *X. glabratus* in southeastern USA (Harrington and Fraedrich 2010). *Raffaelea* species may compete for entrance to and growth within the mycangium. The fact that most *X. glabratus* in USA breed in dead and dying redbay trees already colonized by *R. lauricola* may explain the dominance of this *Raffaelea* species in the mycangia (Harrington and Fraedrich 2010).

Ambrosia beetles generally are attracted to alcohols and other odors emanating from dead and dying trees, and ethanol often is used as an attractant for trapping beetles (Ranger et al. 2010). However *X. glabratus* is not attracted to ethanol but instead is attracted to host plant volatiles (Hanula et al. 2008, Hanula and Sullivan 2008). This attraction to host volatiles is believed to be important in its aborted attacks on live trees, thus providing the opportunity to introduce *R. lauricola* into healthy, susceptible plant hosts (Fraedrich et al. 2008). *R. lauricola* is unusual in that it is the only ambrosia beetle symbiont known to cause a lethal vascular wilt disease (Fraedrich et al. 2008; Harrington et al. 2008, 2010), and the association of *X. glabratus* with a systemic plant pathogen and its attraction to living trees may be related. Thus *R. lauricola* may serve directly as food for *X. glabratus* and also may provide suitable brood material for the beetle by killing trees (Fraedrich et al. 2008). However little is known of the biology of *X. glabratus* in Asia and no study heretofore has examined fungal symbionts of the beetle there.

To determine whether *R. lauricola* and other symbionts of *X. glabratus* were introduced to USA from Asia, we isolated *Raffaelea* species from adult females trapped in Taiwan and Japan with a dilution plating technique (Harrington 1992, Harrington and Fraedrich 2010) to count the number of CFUs of fungal symbionts in adult females of the redbay ambrosia beetle. We conducted isolations from similarly trapped beetles in Georgia to compare the incidence of the *Raffaelea* spp. in a USA population with the beetle populations in Taiwan and Japan.

MATERIALS AND METHODS

Beetle collection.—Adult, female *X. glabratus* were collected at three sites in Taiwan, two sites in Japan and one site in Georgia in 2009 (TABLE I). The sticky traps (wing style bottoms, Scentry Biologicals Inc., Billings, Montana) were baited with manuka oil lures (P385-Lure M; Synergy Semiochemicals Corp., Burnaby, British Columbia) (Hanula and Sullivan 2008). Males of *Xyleborus* species are much smaller than females, are generally flightless and typically mate with their siblings before the young females emerge (Rabaglia et al. 2006). Therefore only female beetles serve as vectors of *R. lauricola* (Harrington and Fraedrich 2010) and only female beetles were trapped.

The Taiwan sites were on property of the Taiwan Forest Research Institute at 600–700 m in broadleaf evergreen forests dominated by Fagaceae and Lauraceae. Dominant tree species at the Fushan Research Center (northern Taiwan) include *Castanopsis carlesii* (Hemsl.) Hayata (Fagaceae), *Engelhardtia roxburghiana* Wall. (Juglandaceae), *Machilus zuihoensis* Hay. (Lauraceae), *Meliosma squamulata* Hance (Sabiaceae) and *Litsea acuminata* (Blume) Kurata (Lauraceae). The Hsinshien Nursery (northern Taiwan) is surrounded by similar forest vegetation, but there was an adjacent plantation of *Cinnamomum osmophloeum* Kanehira (Lauraceae) and *Castanopsis fargesii* Fr. Dominant tree species at the Lienhuachih Research Center (central Taiwan) include *Schefflera octophylla* (Lour.) Harms (Araliaceae) and a number of Lauraceae: *Cryptocarya chinensis* Hemsl., *Actinodaphne nantoensis* Hay., *Neolitsea variabilissima* (Hay.) Kaneh. & Sasaki and *Cinnamomum randaiense* Hay. Some *Machilus* (*Persea*) spp. also occur in these forests.

A sticky trap was set up 1.2–1.5 m above the ground at three locations at the Fushan site, three locations at the Lienhuachih site and two locations at the Hsinshien site Apr–Dec 2009. Traps were covered by a plastic roof to avoid direct rainfall. We checked traps at 2–4 wk intervals, but we left some traps in the field longer. The *X. glabratus* beetles that had adhered to the sticky material on the trap surface (Stickem®, Seabright Laboratories, Emeryville, California) were collected and placed individually in glass or plastic tubes in the field. The beetles were kept at 4–5 C until shipping to Iowa for isolations.

In Japan beetles were collected in broadleaf evergreen forests in Minami-Kyushu City and Satsuma City on Kyushu Island. The Taneo site in Minami-Kyushu City was at 400–500 m in a forest dominated by *Lithocarpus edulis* Nakai (Fagaceae) with some large *Cinnamomum tenuifolium* Sugimoto. The Koshi plot in Satsuma City was at 100–200 m in a forest of nearly pure *Quercus gilva* Blume (Fagaceae), but the plot was near a coniferous plantation of *Cryptomeria japonica* (L. f.) D. Don and evergreen forest of *L. edulis* and *Castanopsis sieboldii* (Makino) Hatus. Beetles were trapped as in Taiwan and shipped to Iowa for processing.

Beetles were collected similarly on three baited sticky traps placed at 2 m above ground in a stand of diseased redbay at Jekyll Island, Georgia. The traps were collected and stored at 4 C until individual beetles were plated in

TABLE I. Number of successful isolations (and range of CFUs per beetle) of *Raffaelea* and *Ophiostoma* species isolated from individual female *Xyleborus glabratus* adults collected at three sites in Taiwan, two sites in Japan and one site in Georgia, USA, in 2009

Country	Location	Dates traps exposed in the field	Date plated	No. of beetles sampled	Beetles yielding no <i>Raffaelea</i> or <i>Ophiostoma</i> sp.	<i>R. lauricola</i>
Taiwan	Lienhuachih	15 May–3 Jun	17 Jun	1	0	1 (2)
		Fushan	5 May–9 Jun	7 Jul	20	9
	Hsinshien	9 Jun–30 Jun	6 Jul	4	2	2 (1–4)
		4 Aug–20 Aug	2 Oct	8	5	3 (40–393)
		20 Aug–16 Sep	2 Nov	15	1	13 (3–3600)
		11 Aug–26 Aug	2 Nov	7	4	3 (3–600)
		26 Aug–11 Sep	21 Sep	20	2	18 (3–9962)
11 Sep–14 Oct	4 Nov	10	2	8 (3–2233)		
Japan	Taneo	17 Aug–25 Sep	29 Sep	1	1	0
	Koshi	17 Jul–17 Aug	29 Sep	6	1	5 (23–3600)
		17 Aug–25 Sep	29 Sep	9	4	5 (10–140)
USA	Georgia	28 Jul–10 Sep	23 Sep	2	0	2 (7–200)
		10 Sep–2 Oct	13–20 Oct	25	13	12 (10–3160)

Georgia (first collection) or placed in tubes and shipped to Iowa for plating (second collection).

Once they arrived in Iowa the beetles were stored at 4 C until processing. Usually the beetles were plated within 2 mo from the time that the traps were placed in the forest, but times between trap placement and plating varied greatly (TABLE I).

Grinding and dilution plating.—A dilution plating technique (Harrington 1992, Harrington and Fraedrich 2010) was used to quantify the number of CFUs per female beetle on CSMA medium (1.0% malt extract and 1.5% agar, with 200 ppm cycloheximide and 100 ppm streptomycin sulfate added after autoclaving), which is semiselective for *Ophiostoma* species and related anamorphs such as *Raffaelea* (Harrington 1981, 1992; Harrington and Fraedrich 2010). A whole adult female was ground in 2 mL glass tissue grinders (Kontes brand) with 200 μ L sterile deionized water (SDW) for 5 min, and another 800 μ L SDW was added, with grinding an additional 5 min. After grinding, 100 μ L of the slurry was diluted with another 900 μ L SDW. A 100 μ L aliquot of the ground slurry (10 \times dilution plates) or the 10-fold dilution (100 \times dilution plates) was added to the surface of the agar medium in 100 mm Petri plates and spread with a flame-sterilized, bent glass rod. The 100 μ L aliquots of slurry or dilution of each beetle was added separately to three Petri plates, except for the Lienhuachih and the first two Fushan collections, for which up to eight Petri plates were used per beetle.

After each use, the inside of the glass tissue grinder was rinsed with hexane to remove residual Stickem[®], rinsed further in 95% ethanol to remove the hexane residue and then in SDW to remove ethanol residue. The grinders were autoclaved before reuse.

Identification and quantification of CFUs.—We examined Petri plates 1–4 wk after plating for the presence of fungal colonies. Colonies of different size, color and mycelial pattern (concentric rings, yeasty centers, hyaline margins etc.) were considered putative species. If the number of

colonies of a putative species on the 10 \times dilution plates exceeded 25 per plate, then 100 \times dilution plates were used for counting colonies. Subcultures of colonies of different morphologies were made to identify the putative species.

CFUs for each putative fungal species were expressed as a mean of the 3–8 plates of the appropriate dilution, and the mean was considered a single replicate. The range of CFUs per beetle was determined for each isolated species in each population (collection). The mean and the standard error of the mean (SE, standard deviation divided by the square root of the number of beetles in the sample) of the CFUs per beetle yielding a particular fungus species were calculated for each country. The proportion of beetles yielding a fungus species from the three countries was compared by the chi-squared test.

Characterization of isolated species.—Species of *Raffaelea* are difficult to identify based on morphology and physiology, so identifications are based primarily on rDNA sequences (Harrington et al. 2010). Unfortunately the PCR amplification and sequencing of the internal transcribed spacer region (ITS-rDNA) of some *Raffaelea* and *Ophiostoma* species with *Leptographium* anamorphs is not routinely possible. Currently, the only complete and sufficiently variable dataset of DNA sequences for identification of *Raffaelea* species is the large subunit (LSU, 26S) rDNA dataset (Harrington et al. 2010).

Sequencing of a portion of the LSU was attempted on at least one representative of each colony morphotype (putative species) from each beetle. Isolates were grown on malt yeast extract agar (MYEA; 2% malt extract, 0.2% yeast extract and 2% agar) 7–14 d at room temperature, and DNA was extracted from the mycelia and spores from the surface of the plates with PrepMan[™] Ultra following the manufacturer's instructions (Applied Biosystems, Foster City, California). LSU was amplified with primers LROR and LR5, and PCR products were sequenced with primers LROR and LR3 (Harrington et al. 2010)

TABLE I. Extended

<i>R. ellipticospora</i>	<i>R. fusca</i>	<i>R. subfusca</i>	Near <i>R. canadensis</i>	Near <i>R. sulphurea</i>	Near <i>R. albimanens</i>	<i>Ophiostoma</i> sp. A	<i>Ophiostoma</i> sp. B	<i>Ophiostoma</i> sp. C
1 (48)	0	0	0	0	0	1 (5)	1 (2)	0
3 (1–16)	0	0	0	0	1 (5)	0	0	4 (2–39)
2 (4–9)	0	0	0	0	0	1 (5)	1 (1)	0
0	0	0	0	0	0	0	0	0
7 (3–43)	3 (17–37)	1 (17)	0	1 (394)	0	2 (10–223)	0	0
1 (40)	0	0	1 (3)	0	0	0	0	0
10 (3–170)	0	0	2 (7–67)	0	0	1 (10)	0	0
2 (10–27)	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
1 (110)	0	0	0	0	0	0	0	0
1 (7)	0	0	0	0	0	0	0	0
1 (147)	0	1 (1913)	0	0	0	0	0	0
6 (10–337)	0	0	0	0	0	0	0	0

Automated sequencing was performed with a DNA Analyzer (DNA Analyzer 3730xl, Applied Biosystems) at the Iowa State University DNA Sequencing and Synthesis Facility (Ames). Comparisons to other LSU sequences were conducted with BLAST queries (2.2.24, National Center for Biotechnology Information, National Institute of Health, Bethesda, Maryland) and a large dataset of LSU sequences of *Ophiostoma* and *Raffaelea* species (Harrington unpubl). Representative sequences of *Raffaelea* spp. and *Ophiostoma* spp. from the isolated species were deposited in GenBank.

Cultures of putative *Raffaelea* species were grown on malt extract agar (MEA, 1% Difco malt extract and 1.5% agar) at 25 C in a dark incubator, along with isolates from *X. glabratus* collected in USA (Harrington et al. 2010, Harrington and Fraedrich 2010). In addition to the extent of growth after 10 d, we noted the color and texture of mycelium, as well as characteristics of conidia, conidiophores, sporodochia or other fruiting structures. Colors reported for mycelia are from Rayner (1970). Representative cultures of the species are maintained in the collection at Iowa State University (C numbers).

RESULTS

Ten cycloheximide-tolerant species with LSU sequences typical of *Ophiostoma* species and their anamorphs were recovered from beetles collected in Taiwan (TABLE I). Seven of the species had LSU sequences near those of described species of *Raffaelea*, and three other species had sequences similar to other members of genus *Ophiostoma*. Only two species, *R. lauricola* and *R. ellipticospora* T.C. Harr., Aghayeva, & Fraedrich, were recovered from beetles collected in Japan. Beetles collected in USA yielded *R. lauricola*, *R. ellipticospora* and *R. subfusca* T.C. Harr., Aghayeva, & Fraedrich. Twenty-five of the 85

beetles collected in Taiwan, six of 16 beetles collected in Japan and 13 of 27 beetles collected in USA yielded no *Raffaelea* or *Ophiostoma* species (TABLE I).

Raffaelea lauricola.—This was the most commonly recovered species from beetles collected in Taiwan, Japan and USA. It was recovered from 56 of 85 beetles sampled from Taiwan, where it was recovered from beetles collected at all three sites at up to an estimated 9962 CFUs from a single beetle (TABLE I). The pathogen was recovered at a mean and SE of 712 ± 193 CFUs per beetle from the 56 beetles. *R. lauricola* was isolated from 10 of 16 beetles sampled from Japan, at up to 3600 CFUs from a single beetle (TABLE I) and a mean of 632 ± 360 CFUs per beetle. The pathogen was isolated from 14 of 27 beetles collected in USA, at up to 3160 CFUs per beetle and a mean of 540 ± 304 CFUs per beetle. There was no difference among the countries in the proportion of beetles yielding *R. lauricola* according to the chi-squared test ($P = 0.4229$, degrees of freedom = 2). No other *Raffaelea* or *Ophiostoma* species was recovered in numbers greater than 400 CFUs from a beetle collected in Taiwan or Japan, but one beetle collected in USA yielded *R. subfusca* at 1913 CFUs.

Isolates identified as *R. lauricola* from Taiwan (C2646 = CBS129006 and C2540 = CBS129001) and Japan (C2664 = CBS129007) had the same distinctive colony morphology and color (white), including a characteristic mucoid growth, as *R. lauricola* cultures from USA studied by Harrington et al. (2008). LSU sequences of most *R. lauricola* isolates from Taiwan and Japan showed a single base substitution at position 507 of the 545 bp of the

deposited sequences for USA isolates of *R. lauricola* (GenBank EU177438–EU177440). A total of 77 LSU sequences from 77 isolates of *R. lauricola* from South Carolina, Georgia, Florida and Mississippi were available, and each of these sequences was identical. These 77 included isolates from *Persea borbonia*, *P. palustris*, *P. americana*, *Sassafras albidum*, *Cinnamomum camphora* L.Sieb., *Lindera melissifolia* Blume, *Litsea aestivalis* (L.) Fernald. and from ambrosia beetles *X. glabratus*, *Xyleborinus saxeseni* (Ratzeburg) and *Xylosandrus crassiusculus* (Motschulsky). Of the 43 isolates of *R. lauricola* sequenced from Taiwan, four (e.g. isolate C2646, sequence HQ688666) had the sequence of the isolates from USA, and 39 isolates (e.g. C2540, HQ688667) had a G substituted for an A at the variable position. Among the 16 isolates sequenced from Japan, three (e.g. C2664, HQ688668) had the sequence of the USA type and 13 had the G substitution typical of the Taiwan isolates.

Raffaelea ellipticospora.—This was the second most frequently isolated species from beetles collected in Taiwan, Japan and USA. It was recovered from 26 beetles collected in Taiwan, two beetles collected in Japan and six beetles collected in the USA at a maximum estimated 170, 110 and 337 CFUs per beetle, respectively (TABLE I). No difference was found among the countries in the proportion of beetles yielding *R. ellipticospora* according to the chi-squared test ($P = 0.3243$, degrees of freedom = 2). The mean and SE for the CFUs per beetle was 24 ± 7 , 58 ± 52 and 119 ± 28 from the 26, two and seven beetles yielding *R. ellipticospora* from Taiwan, Japan and USA, respectively.

The isolates of *R. ellipticospora* from Taiwan and Japan showed a range of LSU sequences, growth rate and colony morphology. Isolates of *R. ellipticospora* from USA collected by Harrington et al. (2010) were mostly gray olivaceous (107) to olivaceous gray (23^{mm}) and 17–27 mm diam on MEA after 10 d. Taiwanese isolates were mostly olive buff (21^{mm}d) to grayish olive (21^{mm}) and 30–33 mm diam after 10 d, although several isolates were smoke gray (21^{mm}d) and 22–26 mm diam at 10 d. Japanese isolates were dark olivaceous gray (23^{mm}I) to isabelline (9^I) and 22–30 mm diam after 10 d on MEA. Sequences of the LSU of the Taiwanese isolates (e.g. C2550 = CBS129002 and C2709 = CBS129008, HQ688663 and HQ688664, respectively) and Japanese isolates (e.g. C2607 = CBS129005 and C2737, HQ688661 and HQ688662, respectively) differed from the LSU sequence of USA isolates of *R. ellipticospora* (EU177444–EU177446) by 1–4 bp. However these minor differences in LSU sequences among Taiwan

and Japan isolates did not correlate with variation in colony color or growth rate.

Raffaelea fusca.—A fungus with a LSU sequence near that of *R. fusca* T.C. Harr., Aghayeva, & Fraedrich was isolated from three beetles collected from the Fushan site (TABLE I). These isolates had a LSU sequence (e.g. C2717 = CBS129011, HQ688658) that differed from USA isolates of *R. fusca* (EU177447–EU177449) by four bp.

Raffaelea subfusca.—A fungus with a LSU sequence (C2712 = CBS129010, HQ688659) near that of *R. subfusca* was isolated from a single beetle collected from the Fushan Research Center (TABLE I). The LSU sequence of this isolate differed from that of USA isolates of *R. subfusca* (EU177450, EU177451, EU177473) by five bp.

Unidentified Raffaelea spp.—A species with LSU sequences (HQ688665) similar to those of *R. canadensis* L.R. Batra (EU177458, 541 of 552 bp matching) was isolated from three beetles collected in Taiwan (TABLE I). One of the beetles had an estimated 67 CFUs of this *Raffaelea* sp., which formed sprout cells and sporodochia in culture. After *R. canadensis* the next closest match for the LSU sequence of this Taiwan species (C2711 = CBS129009) was *R. subalba* T.C. Harr., Aghayeva, & Fraedrich (EU177441–EU177443) with 525 of 552 bp matching.

A species isolated from a single beetle at Fushan (TABLE I) (C2579 = CBS129004) formed sporodochia and sprout cells and had a LSU sequence (HQ688667) somewhat near that of *R. sulphurea* (L.R. Batra) T.C. Harr. (EU177463, 513 of 547 bp matching), *R. monteyi* M. Morelet (EU177461 508 of 548 bp matching), *R. amasae* (Gebhardt) T.C. Harr. (EU984295, 498 of 535 bp matching) and *R. quercivora* Kubuno & Shin. Ito (AB552937, 494 of 528 bp matching).

Another species with sporodochia and sprout cells was isolated (C2569 = CBS129003) from a single beetle at Fushan (TABLE I), and it had a LSU sequence (HQ688657) near that of *R. albimanens* D.B. Scott & J.W. du Toit (EU177452, 537 of 549 bp matching), *R. subalba* (EU177441–EU177443, 534 of 550 bp matching) and *R. santoroi* Guerrero (EU984302, 525 of 539 bp matching).

Unidentified Ophiostoma spp.—Three cycloheximide-tolerant species had LSU sequences that placed them in the genus *Ophiostoma* but not in *Raffaelea*. They did not form sporodochia in culture, but two species formed perithecia and ascospores typical of *Ophiostoma* species.

Ophiostoma sp. A was recovered from five beetles at three sites in Taiwan, and one of the beetles had an estimated 223 CFUs (TABLE I). It had white mycelia, a *Hyalorhinochlaediella* anamorph, and two isolates formed protoperithecia in culture. The protoperithecia did not develop necks unless paired with another isolate of the same species (C2561 × C2715 and C2543 × C2544). Ascospores had rectangular sheaths. We saw no sprout cells or sporodochia typical of *Raffaelea* species in cultures of this *Ophiostoma* species. The LSU sequence (HQ688654 from isolate C2544) did not closely match available LSU sequences but most closely matched that of *R. subalba* (EU177441–EU177443, 509 of 550 bp matching) and a species described (Ohtaka et al. 2006) as *O. rectangulosporum* Ohtaka, Masuya & Yamaoka (AB235158, 499 of 539 bp matching).

Ophiostoma sp. B (e.g. C2542) had light brown mycelia and a *Hyalorhinochlaediella* anamorph, and the conidiophores sometimes formed compact masses resembling synnema. It was isolated from two beetles at low CFUs (TABLE I). Its LSU sequence (HQ688655) most closely matched *O. stenoceras* (Robak Melin & Nannf. (DQ836904, 514 of 537 bp matching) and *O. nigrocarpum* (R.W. Davidson) de Hoog (EF506941, 504 of 527 bp matching).

Ophiostoma sp. C was isolated from four beetles in one batch collected at the Fushan site (TABLE I). It (e.g. C2563) formed dark brown mycelia, *Hyalorhinochlaediella*-like conidiophores with prominent scars from conidial dehiscence and perithecia with half-moon shaped ascospores. The LSU sequence of this species (HQ688656) most closely matched that of *Sporothrix schenckii* Hektoen & C.F. Perkins (FJ827769, 510 of 533 bp matching), *Fragosphaeria purpurea* Shear (AF096191, 512 of 540 bp matching) and *O. stenoceras* (DQ836904, 511 of 540 bp matching).

DISCUSSION

The laurel wilt pathogen was the predominant *Raffaelea* species isolated from *X. glabratus* in Taiwan and Japan, consistent with the results of isolations from this beetle in USA. The percentage of Asian beetles yielding *R. lauricola* and the estimated CFUs per beetle were lower than from fresh beetles collected in South Carolina and Georgia (Harrington and Fraedrich 2010), but some of the beetles in Taiwan and Japan were exposed on sticky traps for a month before shipping, and some isolations were done 3 mo after the traps first were exposed in the field. Isolations from *X. glabratus* collected on sticky traps in Georgia yielded *R. lauricola* in amounts similar to those found in *X. glabratus* from Taiwan

and Japan and less than in fresh beetles without Stickem, which may inhibit fungal survival and impede extraction of spores from the mycangium. Nonetheless *R. lauricola* was isolated from more than half the beetles from Taiwan and Japan and in thousands of CFUs per beetle. These results support the hypothesis that *R. lauricola* was introduced to USA with *X. glabratus* in solid wood packing material from Asia (Fraedrich et al. 2008, Harrington et al. 2008) and suggest that *R. lauricola* is an important symbiont of this beetle in Asia.

Although there has been some suggestion of pathogenicity to plants by other *Raffaelea* species associated with ambrosia beetles that mass-attack stressed trees (Takahashi et al. 2010), no other aggressive plant pathogen capable of killing trees has been associated with an ambrosia beetle (Harrington et al. 2008, 2010). With typical ambrosia beetles, attraction to stressed trees is aided by the attraction of the beetles to ethanol and other compounds emanating from dead and dying trees (Ranger et al. 2010). *X. glabratus* is unusual in its attraction to healthy trees, and *R. lauricola* is unique in its capability of killing a healthy tree from just a single infection (Fraedrich et al. 2008). In USA such infections apparently are aided by aborted attacks by *X. glabratus* in branches or the main stem (Fraedrich et al. 2008), and the attraction of *X. glabratus* to host volatiles emanating from a wounded or healthy trees, instead of ethanol (Hanula et al. 2008, Hanula and Sullivan 2008), is believed to increase the chances of a beetle boring into a healthy, susceptible tree (Fraedrich et al. 2008).

Now that it has been confirmed that *R. lauricola* is the primary symbiont within the native range of *X. glabratus* it is logical to speculate that *R. lauricola* aids *X. glabratus* in Asia by serving as a food source and by providing brood material. The recorded hosts of *X. glabratus* in Asia are such aromatic species as *Lindera latifolia* Hook. f. and *Litsea elongata* Benth. & Hook. f. in the Lauraceae and *Shorea robusta* Gaertn.f. in the Dipterocarpaceae (Wood and Bright 1992). One of us (Lu unpubl) has collected *X. glabratus* from logs of *Cinnamomum osmophloeum* in Taiwan. Although laurel wilt has not been observed in Asia, the Asian species *C. camphora* (camphor tree) is susceptible to laurel wilt in southeastern USA (Smith et al. 2009), although the pathogen might not be able to colonize and kill the whole tree. Instead, infected camphor trees in southeastern USA apparently suffer a branch dieback disease, which might provide suitable brood material for ambrosia beetles. One camphor tree taller than 6 m in Georgia had crown dieback and isolated areas of vascular discoloration in the main stem down to 2 m above ground and *R. lauricola* was

isolated from the discolored xylem (S. Fraedrich unpubl). Affected camphor trees have had ambrosia beetle tunnels of a diameter typical for those made by *X. glabratus*, and a bolt of camphor tree left exposed at Jekyll Island in Georgia in Sep 2009 was attacked by *X. glabratus* (S. Fraedrich unpubl).

Isolates of *R. lauricola* from USA, Taiwan and Japan are similar in culture morphology and growth rate, but there is a single base substitution in the sequenced portion of the LSU. All isolates studied from USA have had the same LSU sequence, and some of the isolates from Taiwan and Japan also have this sequence. However the majority of the Taiwan and Japanese isolates differ at a single base, a substitution from an A to a G. Such minor sequence variation might be common for natural populations of *Raffaelea* species, although there has been limited study of phenotypic and genetic variation in ambrosia beetle symbionts. In another *Raffaelea* species native to Japan, *R. quercivora* Kubono & Shin. Ito, a sampling of 23 isolates showed that four had a single base substitution in the sequenced LSU region (Matsuda et al. 2010).

R. ellipticospora was recovered with relatively low CFUs, suggesting that it has relatively few spores in the mycangia compared to *R. lauricola*. In southeastern USA populations of *X. glabratus*, *R. ellipticospora* is the second or third most commonly isolated fungus, behind *R. lauricola* and sometimes *R. subalba* (Harrington and Fraedrich 2010). In our isolations from *X. glabratus* collected on sticky traps in Georgia, *R. ellipticospora* was isolated in numbers similar to those for beetles collected in Taiwan and Japan. Isolates from Taiwan and Japan were variable in culture morphology and growth rate, and the LSU sequences of the Asian isolates differed by at least one bp from those of USA isolates. *R. ellipticospora* might prove to be a complex of closely related species.

Two other *Raffaelea* species, *R. fusca* and *R. subfusca*, were isolated from one Taiwanese batch of *X. glabratus* at low CFUs per beetle. These species have been isolated occasionally from *X. glabratus* in southeastern USA (Harrington and Fraedrich 2010), and *R. subfusca* was isolated from one beetle collected on a sticky trap in Georgia. Growth rate and pigmentation of cultures of the Taiwan isolates of *R. fusca* and *R. subfusca* differed somewhat from USA isolates of these two closely related species (Harrington et al. 2010), and the LSU sequences of the Taiwan isolates differed by up to four bp from respective species in USA. Isolations from Taiwan bark beetles suggest that *R. fusca* and *R. subfusca* also were introduced to USA from Asia by *X. glabratus*.

Three unidentified *Raffaelea* species were isolated rarely from *X. glabratus* in Taiwan but have not been

found in USA populations (Harrington and Fraedrich 2010). A species with a LSU sequence near that of *R. sulphurea* and a species with a LSU sequence near that of *R. albimanens* each were isolated from a single beetle collected at Fushan, and a fungus with a LSU sequence near that of *R. canadensis* was isolated from three beetles collected at Hsinshien. These three unidentified species formed sporodochia with *Hyalorhinochlaediella*-like conidiophores in culture, further suggesting that they are undescribed ambrosia beetle symbionts.

Three other cycloheximide-tolerant species (*Ophiostoma* sp. A, B and C) were isolated occasionally from beetles collected in Taiwan. In contrast to the asexual *Raffaelea* spp. (Harrington et al. 2010), two of the Taiwan species formed perithecia typical of *Ophiostoma* in culture and none of the three species formed sporodochia typical of *Raffaelea* when cultured on MEA or MYEA. To date a sexual state has not been found for any ambrosia beetle symbiont, and a sexual state would not likely be an advantage for dispersal by an ambrosia beetle with mycangia (Harrington 2005, Harrington et al. 2010). However *Ophiostoma* spp. and molds such as *Fusarium* spp. commonly produce spores in older galleries of ambrosia beetles (Batra 1963, Harrington 2005). It is likely that the three *Ophiostoma* spp. were external contaminants of the beetles and not mycangial symbionts.

Two *Raffaelea* species have been isolated from *X. glabratus* in southeastern USA but were not isolated from the beetles in Taiwan or Japan. *R. arxii* was isolated from four beetles in Georgia but only with low CFUs (Harrington and Fraedrich 2010). This species also was isolated from *Xyleborus torquatus* Eichh. in South Africa (Harrington et al. 2010), and it is possible that the species is a symbiont of another ambrosia beetle introduced to the USA.

In contrast, *R. subalba* was isolated relatively frequently from beetles in Georgia and South Carolina, with more than 5000 CFUs per beetle (Harrington and Fraedrich 2010). The absence of *R. subalba* in the Taiwan and Japan populations is somewhat surprising and might indicate that *X. glabratus* acquired this symbiont in USA. On the other hand *R. subalba* might be a symbiont of *X. glabratus* in another part of Asia, perhaps where the USA population originated. Based on similarity of LSU sequences (Harrington et al. 2010, Massoumi Alamouti et al. 2009), the Taiwan species referred to here as “near *R. albimanens*” might be part of a species complex that includes *R. subalba*, *R. albimanens* (from *Platypus externedentatus* Fairm. in South Africa), *R. sulcati* A. Funk (from *Gnathotrichus sulcati* Lec. in Canada), *R. santoroi* (from *Platypus* sp. in

Argentina) and *R. tritirachium* L.R. Batra (from *Monarctum mali* (Fitch) in Pennsylvania). Much more work is needed in studying genetic variation and species limits of fungal symbionts of ambrosia beetles and other asexual fungi (Harrington and Rizzo 1999).

There have been relatively few qualitative or quantitative studies of fungal associates of ambrosia beetles (Harrington and Fraedrich 2010, Kinuura 2002), and the biology of relatively few ambrosia beetles has been studied in detail (Harrington 2005). Hulcr and Cognato (2010) reported that some ambrosia beetles do not have mycangia to carry symbionts but instead habitually steal their symbionts from the nearby galleries of other ambrosia beetle species. Before work on *X. glabratus* ambrosia beetles generally were thought to be tightly associated with one or only a few symbiotic fungi (Batra 1963, 1967; Funk 1970), but recent studies suggest that the symbionts of ambrosia beetles are more diverse, more promiscuous and more competitive than previously assumed (Harrington et al. 2010, Harrington and Fraedrich 2010). In addition to the six *Raffaelea* species isolated from *X. glabratus* in USA (Harrington and Fraedrich 2010) at least three undescribed *Raffaelea* species were isolated from *X. glabratus* in Taiwan, and there are indications that many more species will be isolated from *X. glabratus* collected in other parts of Asia.

Although this study confirms that *R. lauricola* is native to Asia and was introduced to southeastern USA with *X. glabratus*, it does not necessarily indicate that it was introduced from Taiwan or Japan. *X. glabratus* also has been found in India and Myanmar (Wood and Bright 1992), and the beetle is likely present but undocumented in other southeastern Asian countries. Differences in LSU sequence, mycelial pigmentation and growth rate among isolates of *R. ellipticospora*, *R. fusca* and *R. subfusca* suggest that it is more likely that we have not yet sampled the Asian population of *X. glabratus* from where the USA population originated. The identity of the source of the USA population is particularly important because an introduction of *X. glabratus* and *R. lauricola* to another region of the world dominated by Lauraceae could prove to be even more devastating than the introduction to southeastern USA (Fraedrich et al. 2008).

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