Biosecurity capacity building for the Australian avocado industry: Laurel Wilt

Dr Andrew Geering
The Department of Agriculture, Fisheries and Forestry, QLD

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Biosecurity Capacity Building for the Australian Avocado Industry: Laurel Wilt

By Andrew D. W. Geering and Paul R. Campbell

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Project leader's name and contact details:

Dr Andrew D.W. Geering (Senior Research Fellow, Queensland Alliance for Agriculture and Food Innovation, The University of Queensland).

Postal address - GPO Box 267, Brisbane, QLD 4001.
Street address - Level 2C Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4001.
Phone - (07) 32554389
Email - a.geering@uq.edu.au

Other key personnel:

Dr Paul R. Campbell (Plant Pathologist, Queensland Department of Agriculture, Fisheries and Forestry).

This final report describes the findings of the project "Biosecurity Capacity Building for the Australian Avocado Industry: Laurel Wilt"

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1. Media Summary

Ambrosia beetles share one special feature with humans, the ability to farm. These weevil-sized beetles colonize wood but rather than consuming this rather nutrient-poor material, they inoculate the tree with a particular type of fungus, which then becomes the food for the beetle larvae. Many tree species have evolved specialized chemical defences against this type of insect infestation and normally the fungal symbiont is only weakly pathogenic. However, there are some notable exceptions to this rule where perfectly healthy trees have succumbed to ambrosia beetle infestation and their fungal symbionts have acted as aggressive pathogens. One classic example occurs in the south-eastern states of the USA, where cultivated avocado and its wild relatives are afflicted by a lethal disease called laurel wilt. The culprit is the beetle *Xyleborus glabratus*, which transmits the fungus *Raffaelea lauricola*. *X. glabratus* is native to Asia and entered the USA as a stowaway in wooden crates. *X. glabratus* is considered one of the most serious biosecurity threats to the Australian avocado industry.

In a pre-emptive move to prevent *X. glabratus* entering Australia, scientists from Brisbane have visited their counterparts in the USA to learn about laurel wilt disease. As a result of this collaboration, a diagnostic manual for laurel wilt has been produced, which will become the national standard for Australia. During preparation of this manual, new molecular diagnostic assays for both *X. glabratus* and *R. lauricola* were developed. Armed with these new assays, ambrosia beetle surveys were done in subtropical and tropical avocado production areas of the east coast of Australia and thankfully no *X. glabratus* found, confirming Australia's pest-free status. However, there is no room for complacency, as two more ambrosia beetle species were discovered (*Euwallacea* sp. aff. *fornicatus* and *Microperus* sp.) were found on the Sunshine Coast and Atherton Tablelands in Queensland. Infestations of these beetles were associated with avocado canopy thinning and most likely yield loss. The fungal symbionts of these ambrosia beetles were isolated and shown to be new species in the genera *Fusarium* and *Bionectria*. When pure cultures of these fungi were injected into the stems of young avocado plants, large brown lesions were produced in the sapwood, confirming that these fungi were causing disease.

2. Technical Summary

Laurel wilt disease is regarded as one of the most serious biosecurity threats to the Australian avocado industry. Laurel wilt is caused by the ambrosia beetle *Xyleborus glabratus*, which transmits the fungal pathogen *Raffaelea lauricola*. Although the avocado (*Persea americana*) is not a good reproductive host of *X. glabratus*, one aborted attempt at burrowing into the tree trunk is sufficient to transmit *R. lauricola*, which then can cause rapid wilting and plant death, especially of highly susceptible West Indian genotypes of the species. *X. glabratus* is native to western parts of Asia but has spread to the south-eastern states of the USA, where it is devastating redbay (*Persea borbonia*) populations. In 2010, *X. glabratus* extended its range to Miami-Dade County in Florida, the second largest avocado production area in the USA, and now threatens the viability of this industry.

X. glabratus is thought to have entered the USA as a stowaway in wooden packing material used to transport cargo. Australia is a large importer of manufactured goods from Asia and therefore is at risk of an incursion of *X. glabratus* in the same manner as the USA. Any attempts to eradicate *X. glabratus* from Australia will depend on rapid recognition of an incursion. Research done in this project was aimed at providing this diagnostic capability.

One major output of this project was the preparation of a diagnostic manual for laurel wilt disease. To prepare this manual, it was necessary to travel to the USA to observe the disease in the field, to learn about methods of identification that have been developed there and to collect diagnostic controls that could be used in Australia. The other essential component of preparedness was to validate any diagnostic protocol in Australia to ensure it was capable of discriminating the targets of the assays from the background of endemic ambrosia beetles and fungi.

During the course of this project, new PCR diagnostic assays were developed for both *X. glabratus* and *R. lauricola*. A trapping program for ambrosia beetles was also done to determine what species are typically associated with avocado orchards on the east coast of Australia. The *X. glabratus* and *R. lauricola*-specific PCR assays were tested against those species present in Australia and the specificity of the assays demonstrated, as well as evidence for the absence of the target species provided. However, two other ambrosia beetle species were found infesting diseased avocados, namely *Euwallacea* sp. aff. *fornicatus* and *Microperus* sp.

To determine if the two new ambrosia beetle species were in part responsible for the disease symptoms that were observed or had just opportunistically colonized trees that were diseased because of some other reason, fungal isolations were done from the beetles and from the diseased wood. New *Fusarium* and *Bionectria* spp. were isolated from the *Euwallacea* and *Microperus* spp., respectively. Pure cultures of these fungi were used to inoculate healthy, young avocado plants, and necrotic lesions in the sapwood were produced, suggesting that these fungi were behaving as pathogens.

The diagnostic manual produced in this project has been submitted to the Subcommittee on Plant Health Diagnostic Standards for accreditation as the national standard for Australia. However, to avoid this manual becoming outdated, it will be

necessary to revise the content at least biennially. Although it appears that Australia has so far avoided an incursion of *X. glabratus*, there are already other ambrosia beetle species present that are causing disease in avocado orchards. The economic significance of these beetle species is unknown, and methods of control have not been developed, and further research on these topics is therefore needed.

3. Introduction

Laurel wilt is a disease of plants in the family Lauraceae, particularly members of the genus *Persea* (avocado and its wild relatives). Laurel wilt is caused by the fungus *Raffaelea lauricola*, which has a symbiotic relationship with and is vectored by the ambrosia beetle *Xyleborus glabratus* (Coleoptera: Curculionidae: Scolytinae: Xyleborini) (Fraedrich *et al.* 2007; Fraedrich *et al.* 2008; Harrington *et al.* 2008). Female *X. glabratus* carry the blastospores (yeast cells) of *R. lauricola* in specialized sacs called mycangia, which are located at the base of the insect's mandibles (Fraedrich *et al.* 2008). When a female locates a suitable host tree, it burrows into the sapwood to create a brood gallery and at the same time inoculates the tree with the fungus. The fungus penetrates the xylem and when the beetle's eggs hatch, the larvae feed off the fungal hyphae that grow along the surface of the brood gallery.

In their natural environment, ambrosia beetles such as *X. glabratus* will only colonize stressed and dying trees and they play an important role in forest regeneration. *X. glabratus* originates from Asia, but laurel wilt disease has not been recorded in this region. In Taiwan, only trees that have pre-existing damage from termites or longhorn beetles are colonized by *X. glabratus* (J. Peña, personal communication). Laurel wilt has only emerged as a problem when *X. glabratus* was introduced into south-eastern USA, where several indigenous tree species such as *Persea borbonia* (redbay) and *Persea palustris* (swamp bay) have lethal reactions to infection. Also atypical of ambrosia beetles, *X. glabratus* are attracted to and colonize healthy *Persea* spp.

Ambrosia beetles have a high invasion potential because of their cryptic nature (small size and wood-boring habits) and peculiar breeding system. *X. glabratus*, like all members of the Xyleborini, has a haplodiploid breeding system: the female is diploid (2N) while the male is haploid (N) (Jordal *et al.* 2000). Only a single female is required to establish a colony as an unmated female will produce males, which then mate with their mother to produce the next generation of females. Mating then occurs between siblings in the natal nest and thus, ambrosia beetle populations are clonal lineages. The males are smaller and flightless and do not leave the brood gallery, whereas the females will make short flights to colonize new host trees.

X. glabratus was first recorded in the south-eastern US state of Georgia in 2002. It is thought to have hitchhiked in wooden packing material (crates or pallets) used to transport commercial cargo (Haack 2006; Harmon and Brown 2009). Since its introduction into the USA, *X. glabratus* has spread at an estimated rate of 17-20 miles (32 kilometres) per year through natural reproduction and has made much larger jumps as a result of humans transporting infested firewood (Mayfield *et al.* 2008).

At present, *X. glabratus* has not been recorded from Australia and it is regarded as one of the greatest biosecurity threats to the avocado industry in this country. The chance of *X. glabratus* entering Australia would appear even greater than that into America, given our closer geographic proximity to South East Asia, our strong trade links with countries in this region and the sometimes unregulated movement of people and cargo through minor ports. Furthermore, *X. glabratus* is very cryptic and symptoms of laurel wilt difficult to diagnose. In the USA, symptoms were

initially mistaken for those caused by water stress or other diseases. *X. glabratus* entrance holes are small (approximately 0.75 mm in diameter), sometimes rare and found only after intensive searching in the early disease stages (Fraedrich *et al.* 2008). *X. glabratus* is black-brown, small (approximately 2 mm long) and spends most of its lifecycle within host trees (Florida Dept of Ag & Consumer Services).

The capacity of the Australian avocado industry to contain and even eradicate *X. glabratus* and its fungal symbionts will depend on the speed at which an incursion into Australia can be detected. It is thus prudent to have a surveillance and diagnostic capacity in place well in advance of a possible incursion. The major aim of this project was to develop an effective diagnostic capacity for both *R. lauricola* and *X. glabratus*. An essential component of validating the diagnostic assay was to ensure that it was capable of discriminating between the exotic organisms and closely related, endemic organisms. Work that was done in this project will provide an early warning capacity and allow development of a response plan if the organisms breach Australian quarantine.

4. Materials & Methods

4.1 *Reference specimens of* X. glabratus *and* R. lauricola

X. glabratus and *R. lauricola* specimens were kindly donated by Professor R. Ploetz (University of Florida). *X. glabratus* specimens were exported to Australia as corpses soaked in 70% ethanol and DNA from pure agar cultures of *R. lauricola* was extracted in the USA and then exported to Australia in this form.

4.2 *Ambrosia beetle surveys in Australia*

Cross panel sticky traps were fabricated from 5 mm black corrugated plastic. The total height of the traps including the cap was 450 mm and Stikem Special HiTack (Seabright Laboratories, Emeryville, CA, USA) was applied to a *c*. 300 × 100 mm area on each of the eight surfaces of the trap. Two different lures were trialled, ethanol (Alpha Scents, West Linn, OR, USA) and manuka oil (Synergy Semiochemicals Corp., Burnaby, BC, Canada), and control traps without any lure were also deployed. The lures were affixed in the centre of the trap, with a window allowing full dispersal of the scent.

The traps were placed within or next to avocado orchards in three regions, the Atherton Tablelands, Glasshouse Mountains, and Mt Tamborine/Northern NSW. Traps were also placed within feral camphor laurel groves in the Sunshine Coast and northern NSW. A total of nine traps were placed at each location, comprising three of each lure type (unbaited, ethanol, manuka oil). The Atherton tablelands traps were placed in the beginning of September 2011, the other traps were placed in mid November 2011. All traps were left in place for 6 weeks.

Beetles were removed from the sticky trap using a mixture of organic solvent and D-limonene (De-Solv-It, RCR International, Victoria, Australia), then washed with 10 mL of De-Solv-IT to remove residual traces of Stickem, before filtering onto 100 mm filter paper discs. Ambrosia beetles were then collected, washed twice in 10 mL of ethanol and stored at -20 °C in ethanol for later identification and DNA extractions.

4.3 *Fungal isolations*

Avocado trees with evidence of ambrosia beetle colonization were observed on two properties in Queensland (one near Tolga on the Atherton Tablelands and one near Beerwah on the Sunshine Coast). Branches from the infested trees were placed in plastic containers, brought back to Brisbane and stored in the dark until the beetles emerged. The beetles were then surfaced sterilised by washing once for 30 s in 70 % ethanol, followed by two washes in sterile water. The beetle heads were removed with a sterile scalpel blade, and homogenised with a glass tissue grinder in 2 mL of sterile water. Dilutions were then plated on ½ strength potato dextrose agar (PDA) plates containing 100 mg/L streptomycin (PDA+S) and incubated at 25 °C for 48 h. Single colonies were isolated from the dilution plates and replated onto PDA+S plates.

Fungal cultures were also recovered from branches and beetle galleries. The bark was stripped from the outside of the branched, and the wood washed with 1 % sodium hypochlorite. Slivers were cut with a sterile scalpel, and placed on PDA+S plates.

4.4 *DNA extractions*

To isolate beetle DNA, individual beetles were washed twice with 200 µl of ethanol, then twice in 200 µl of deionised water. DNA was then extracted from beetles using the DNeasy Blood & Tissue Kit (QIAGEN) with the following modifications: 90 µl of buffer ATL and 10 µl of Proteinase K were added, then incubated overnight at 56 °C in a shaking block (Thermomixer, Eppendorf). The solution was then removed, into a new tube and the kit directions followed, halving the stated volumes. The beetle cadaver was then washed three times with 200 µl of 70 % ethanol, before storage in 100 % ethanol, and morphological identification.

Fungal DNA was isolated using a BioSprint 15 Plant DNA Kit (QIAGEN). As starting material, two loopfuls of fungal hyphae were placed in a 1.5 mL microcentrifuge tube and ground with a microfuge pestle in 400 µL of RLT Buffer. Following centrifugation at 14,000 rpm for 10 s in a microfuge, 300 µL of the supernatant was collected and the DNA purified following the kit instructions.

4.5 *Molecular identification of ambrosia beetles*

The insect DNA barcode locus, cytochrome c oxidase subunit I (COI), was used for ambrosia beetle identifications. For PCR amplifications, the Jerry/Pat primer pair of Simon *et al*. (1994) and the LCO1490/HCO2198 primer pair of Folmer *et al*. (1994) were used. Two microliters of the DNA extractions were used as template and added to the reagents for the PCR, with final concentrations of 200 µM of dNTPs, 0.125 µM of each primer, 1 × Phusion™ HF buffer, 0.5 Units of Phusion™ DNA Polymerase (New England Biolabs, MA, USA). The PCR was done in a Kyratec SuperCycler thermocycler (Kyratec, Qld, Aust) with the following conditions: an initial denaturation step of 98 °C for 60 s, followed by 35 cycles of 98 °C for 10 s, 50 °C for 15 s, 72 °C for 30 s, and a final extension of 72 °C for 2 min. The PCR products were cleaned up using a QIAquick PCR Purification Kit (QIAGEN) before direct sequencing.

4.6 *Molecular identification of fungi*

Two genetic loci were used for fungal identifications, ITS and TEF1α. The ITS locus was amplified using ITS1F and ITS4 primers (Gardes and Bruns 1993; White *et al.* 1990). The TEF1α locus was amplified using EF-728F and EF2 primers (Carbone and Kohn 1999; O'Donnell *et al.* 2008). Two µL of DNA was used as template and added to the reagents for the PCR, with final concentrations of 200 µM of dNTPs, 0.125 µM of each primer, 1 × Phusion™ HF buffer, 0.5 Units of Phusion™ DNA Polymerase (New England Biolabs, MA, USA). Thermocycling conditions consisted of an initial denaturation step of 98 °C for 60 s, followed by 35 cycles of 98 °C for 10 s, 55 °C for 15 s, 72 °C for 30 s, and a final extension of 72 °C for 2 min.

The PCR products were cleaned up using a QIAquick PCR Purification Kit (QIAGEN) before direct sequencing.

4.7 *Environmental barcoding*

Forty-two individual beetles were used for environmental barcoding (Table 1). Environmental barcoding is the technique whereby DNA is extracted from an environmental sample, DNA barcode PCRs are done and the mixture of barcode sequences is interrogated using massively paralleled sequencing technologies (454 pyrosequencing). Beetle DNA was extracted as per section 4.4, and the barcoding PCRs performed as per section 4.5 and 4.6 except that 5 µL of beetle DNA was used for the fungal ITS and TEF PCRs. Five microliters or each reaction was analysed by gel electrophoresis and the products mixed to allow differential sequencing of the fungal barcodes and the beetle barcodes. The mixed PCR products were cleaned up with QIAquick PCR Purification Kit (QIAGEN) before sending for 454 sequencing. The 454 sequencing was done by Beijing Genomics Institute (BGI – Shenzhen, China). Adapters were ligated onto the ends of PCR products to facilitate the emulsion PCR and sequenced using a GS FLX system (Roche).

Analysis of the 454 sequencing data was through use of QIIME (Caporaso *et al.* 2010) and custom scripts. For the ITS analysis, the Operational Taxonomic Units (OTUs) were picked using uclust_ref using the UNITE/QIIME ITS 12_11 alpha reference OTUs, with a similarity threshold of 0.97. The TEF OTUs were picked through uclust_ref with a custom made reference file containing available TEF sequences, and similarity threshold set to 0.97. Taxonomy for both analyses was assigned using BLAST with e-value of 0.01.

Table 1. Ambrosia beetles used for environmental barcoding

Sample Number	Collection location	Bait	Species
1	Mt Tamborine	Ethanol	*Ambrosiodmus latecompressus*
2	Beerwah	Ethanol	*Ambrosiodmus latecompressus*
3	Mt Tamborine	Ethanol	*Ambrosiodmus latecompressus*
4	Mt Tamborine	Ethanol	*Xyloborinus artelineatus*
5	Mt Tamborine	Ethanol	*Diuncus adossuarius*
6	Mt Tamborine	Ethanol	*Microperus eucalypticus*
7	Mt Tamborine	Ethanol	*Xyleborinus saxesenii*
8	Mt Tamborine	Ethanol	*Xylosandrus morigerus*
9	Mt Tamborine	Ethanol	Unidentified
10	Mt Tamborine	Ethanol	Unidentified
11	Mt Tamborine	Ethanol	Unidentified
12	Mt Tamborine	Ethanol	*Microperus eucalypticus*
13	Beerwah	Ethanol	*Xylopsocus gibbicollis*
14	Mt Tamborine	Ethanol	*Xylopsocus gibbicollis*
15	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
16	Mt Tamborine	Ethanol	*Xylosandrus morigerus*
17	Mt Tamborine	Ethanol	*Xyleborinus saxesinii*
18	Atherton Tablelands	Ethanol	*Xylosandrus dicolor*
19	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
20	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
21	Atherton Tablelands	Not Trapped	*Microperus eucalypticus*
22	Atherton Tablelands	Not Trapped	*Euwallacea fornicatus*
23	Beerwah	Not Trapped	*Euwallacea fornicatus*
24	Florida	Not Trapped	*Xyleborus glabratus*
25	Mt Tamborine	Ethanol	*Ambrosiodmus latecompressus*
26	Mt Tamborine	Ethanol	*Xyloborinus artelineatus*
27	Mt Tamborine	Ethanol	*Xyleborinus saxesenii*
28	Mt Tamborine	Ethanol	*Hypocryphalus mangiferae*
29	Mt Tamborine	Ethanol	*Platypus omnivorus*
30	Mt Tamborine	Manuka	*Wallacellus similis*
31	Mt Tamborine	Manuka	*Hypothenemus sp*
32	Mt Tamborine	Ethanol	*Xylopsocus gibbicollis*
33	Mt Tamborine	Ethanol	*Xylosandrus crassiusculus*
34	Beerwah	Manuka	*Euwallacea fornicatus*
35	Beerwah	Manuka	*Cyclorhipidion pitogenes*
36	Beerwah	No Lure	*Xylosandrus dicolor*
37	Atherton Tablelands	Ethanol	*Xyleborus perforans*
38	Atherton Tablelands	Manuka	*Xyleborinus artelineatus*
39	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
40	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
41	Atherton Tablelands	Ethanol	*Xylopsocus gibbicollis*
42	Atherton Tablelands	Ethanol	*Amasa truncata*

4.8 X. glabratus-*specific PCR assay*

As there is currently no available diagnostic PCR assay for *X. glabratus*, new primers were designed. Ambrosia beetle mitochondrial cytochrome oxidase I (COI)

gene sequences, including that of *X. glabratus*, were downloaded from GenBank, and *X. glabratus*-specific PCR primers designed using in-house software (Table 2).

DNA was extracted from the beetles using the methods described above. PCR mixes contained primers XglabF, XglabR, pUCF16 and pUCR789, all at a final concentration of 100 nM, and 1 unit of *Taq* DNA polymerase (Invitrogen), 1 × *Taq* DNA polymerase buffer (Invitrogen), 200 μM of each dNTP and 2 mM $MgCl_2$ in a final reaction volume of 25 μL. Thermocycling conditions were one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s, and finally, one cycle at 72 °C for 2 min. Eight microlitres of PCR product was separated on a 1.5 % agarose gel in 0.5 × TBE and visualized by staining with ethidium bromide.

4.9 R. lauricola-*specific PCR assay*

A new diagnostic PCR assay for *R. lauricola* was also developed, using primers chosen from the β-tubulin gene sequence of *R. lauricola*. To obtain the relevant gene sequence, the fungal DNA was amplified using the generic primers T1 and T222 as described by O'Donnell and Cigelnik (1997) and the PCR fragment directly sequenced. For comparative purposes, the β-tubulin gene sequence from *Raffaelea* sp. isolate 272 was also obtained. The PCR primers were then selected by eye using an alignment of the two sequences.

PCR mixes contained 100 nM each of RLbtubF and RLbtubR primers, 1 unit of *Taq* DNA polymerase (Invitrogen), 1 × *Taq* DNA polymerase buffer (Invitrogen), 200 μM of each dNTP and 2 mM $MgCl_2$ in a final reaction volume of 25 μL. Thermocycling conditions were one cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s, and finally, one cycle at 72 °C for 2 min. Eight microlitres of PCR product was separated on a 1.5 % agarose gel in 0.5 × TBE and visualized by staining with ethidium bromide.

Table 2. PCR primers used in diagnostic assays

target	Name	Sequence (5'→3')	Amplicon size (bp)
Xyleborus	XglabF	GGAACCTCTCTAAGTGTACTAATTCG	300
glabratus	XglabR	GGTAAACTGTCCATCCCGTTCCTGCT	
pUC19	pUCF19	ATGACGGTGAAAACCTCTGACACATGC	800
	pUCR789	GCTCACATGTTCTTTCCTGCGTTATCC	
Raffaelea	RLbtubF	CTGGTATGTCTCCCAATCCCTTATCTACGC	
lauricola	RLbtubR	GCCAGAGGCCTAGTTATGTCTATTAGATG	

4.10 *Pathogenicity tests*

Bionectria colonies were grown on ½ MEA (2 g/L dried malt extract, 15 g/L agar) media containing 100 mg/L streptomycin. *Fusarium* colonies were grown on Spezieller Nährstoffarmer Agar (SNA). Four 1 cm^2 filter paper squares were placed on each plate, which were then incubated at room temperature under UV light to

enhance sporulation. Conidia were removed from the plates with a sterile loop and washed into 100 µL of water, then diluted to 10^5 colony forming units (cfu)/mL.

Persea americana cv. 'Reed' seedlings and grafted 'Hass' and 'Shepard' plants were obtained from a commercial nursery. Holes (1.5 mm ø) were drilled at an oblique angle into the stems of the seedlings between 20 mm and 50 mm above the graft junction ("Hass" and "Shepard") or the soil ("Reed"). Condia suspensions (10 µL of 10^5 cfu/mL) were pipetted into the holes, which were sealed with Parafilm. Control inoculations were carried out using sterile water. Five plants of each cultivar were used for each treatment for a total of 15 plants. The plants were dissected after 37 weeks post inoculation to determine the extent of internal symptom development.

5. Results

5.1 *Florida study tour*

Although *X. glabratus* and *R. lauricola* originate from South-East Asia, laurel wilt disease symptoms have never been observed in this region. South-eastern USA is so far the only place that laurel wilt has been recorded and therefore to view the disease first-hand and to gain training in its diagnosis, it was necessary for Drs Andrew Geering and Paul Campbell to visit Florida.

A summary of the activities undertaken on the study tour are provided in Table 3. The study tour was hosted by Professor Randy Ploetz, who leads a large multi-faceted project on laurel wilt. Many aspects of laurel wilt biology are being investigated at several research centres in Florida, ranging from fundamental research on beetle biology and the physiology of disease development, through to applied aspects of disease control such as fungicide usage, the deployment of resistant plant varieties and the development of better research extension services. During the two-week itinerary, meetings were held with almost all of the specialists working on the disease and opportunities were provided to observe the disease in the field and to gain 'hands-on' training in disease diagnosis. An immediate output from the study tour was the publication of diagnostic images on the PADIL Web site (http://www.padil.gov.au/pests-and-diseases/Pest/Main/141003).

Table 3. Travel diary for Florida study tour

Date	Location	Contact person	Activity
29 Feb	Miami, FL	Dr Paul Kendra, USDA	Meeting to discuss research project on development of attractants/lures for the redbay ambrosia beetle.
1 Mar	Miami, FL	Dr Ray Schnell, USDA	Meeting to discuss translocation plans for the US avocado germplasm collection from Florida to Hawaii. Walked through avocado germplasm collection, watched tree being injected with fungicide to control laurel wilt, viewed two avocado mapping populations (Hass × Bacon, Simmonds × Tonnage), learnt about marker studies using microsatellite and SNP markers, discussed ASBVd-indexing procedures.
2 Mar	Homestead, FL	Dr Sharon Inch, University of Florida	Meeting to discuss research being done to investigate the cause of wilt symptoms, namely the effect of *R. lauricola* infection on vascular functioning. Experimental apparatus to measure vascular conductance was viewed in operation.
3 Mar	Lake Alfred, FL	Drs Lukasz Stelinski and Jiri Hulcr, University of Florida	Meeting to discuss research project on development of attractants/lures for the redbay ambrosia beetle and to learn about related projects on ambrosia beetle biology and semiochemistry.
4 Mar	Gainesville, FL	Dr Jason Smith, University of Florida	Meetings to discuss research program, including investigations of the efficacy of wood chipping to eliminate beetles, efforts to develop new molecular diagnostic tests for *R. lauricola*, and screening of redbays for resistance to laurel wilt. Visited Austin Cary Forest to view redbay trees in different stages of disease development and to inspect beetle traps.
7-11 Mar 2011	Homestead, FL	Drs Randy Ploetz, Aaron Palmeteer, Jonathan Crane, University of Florida	Training provided in isolation and culturing of *R. lauricola* from the head of *X. glaboratus* and from diseased vascular tissue of a tree. Training provided in technique used to inoculate avocados with *R. lauricola* for pathogenicity testing and resistance screening. Experimentally-inoculated avocados inspected. Discussions with research extension specialists and university plant disease clinic visited.

5.2 *Design of a* X. glabratus *diagnostic assay*

A new PCR primer pair (XglabF and XglabR) targeting the COI gene of *X. glabratus* was designed for use in a diagnostic assay. To test the specificity of the primers, a BLAST search of GenBank was done and no exact matches obtained. The PCR primers were then tested in a duplex assay with the pUC19 internal amplification control and *X. glabratus* template obtained using two different DNA extraction methods. Positive PCR results (amplification of a 300 bp DNA fragment) were obtained for *X. glabratus* using both DNA extraction methods (Figure 1) and no PCR inhibition was observed for any sample.

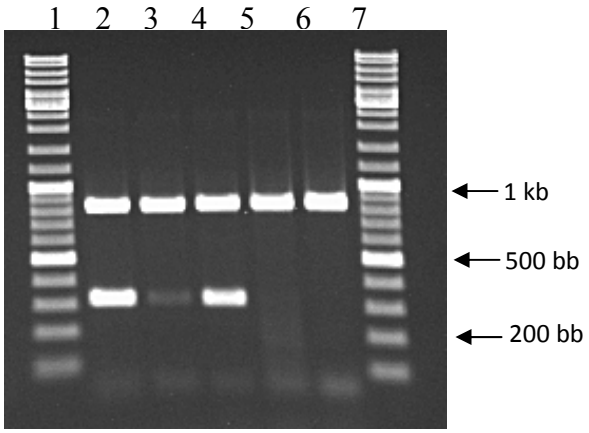

Figure 1. Specific detection of *Xyleborus glabratus* by PCR

The PCR products were ran out on a 1.5% agarose gel in 0.5 × TBE. Order of loading: GeneRuler 100 bp DNA Ladder (lanes 1 and 7), 1:10 dilution of *X. glabratus* DNA (lane 2), 1:100 dilution of *X. glabratus* DNA (lane 3), 1:10 dilution of *X. glabratus* DNA (lane 4), 1:10 dilution of *E. fornicatus* DNA (lane 5), no template control (lane 6). The PCR template for lanes 2, 3 and 5 was prepared using a QIAGEN Biosprint 15 Kit, and that for lane 4 using the TPS soak method.

The primers were then tested against ambrosia beetle species trapped in and around avocado orchards from Queensland and northern NSW (Table 4). DNA extracts were prepared from representative individuals of each species and then 2 µl of DNA extract from five or six species pooled together (Table 4) and a 1 µl aliquot of the combined DNA extract added to a PCR. Pooled sample 3 was duplicated and then spiked with 1 µl of *X. glabratus* DNA extract for use as a positive control (Figure 2). There was no amplification of the 300 bp *X. glabratus*-specific COI fragment for any of the species except for the positive control DNA. There was no PCR inhibition with any sample, even when the DNA extracts were used undiluted, as evident by the positive results for the IAC.

Table 4. Beetle species tested against *X. glabratus*-specific PCR

Higher taxon	Species	Authority	PCR Pool
Scolytinae	*Ambrosiodmus latecompressus*	(Schedl, 1936)	1
Scolytinae	*Xyloborinus artelineatus*	(Beeson, 1929)	1
Scolytinae	*Diuncus adossuarius*	(Schedl, 1952)	1
Scolytinae	*Xyleborinus saxesenii*	(Ratzeburg, 1837)	1
Scolytinae	*Xylosandrus morigerus*	(Blandford, 1894)	1
Scolytinae	*Hypocryphalus mangiferae*	(Stebbing, 1914)	1
Platypodinae	*Platypus omnivorus*	(Lea, 1904)	2
Scolytinae	*Wallacellus similis*	(Ferrari, 1836)	2
Scolytinae	*Hypothenemus* sp.		2
Scolytinae	*Microperus eucalypticus*	(Schedl, 1938)	2
Bostricthidae	*Xylopsocus gibbicollis*	(Macleay, 1873)	2
Scolytinae	*Xylosandrus crassiusculus*	(Motschulskv, 1866)	2
Scolytinae	*Euwallacea fornicatus*	(Eichhoff, 1868)	3
Scolytinae	*Cyclorhipidion pitogenes*	(Schedl, 1936)	3
Scolytinae	*Xylosandrus dicolor*	(Blanford, 1898)	3
Scolytinae	*Xyleborus perforans*	(Wollaston, 1857)	3
Scolytinae	*Amasa truncata*	(Erichson, 1842)	3

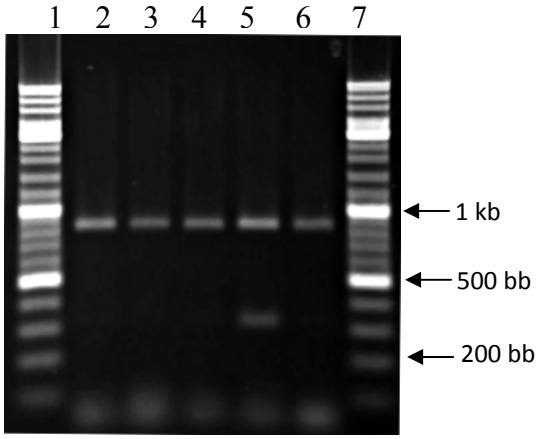

Figure 2. Specificity testing of *Xyleborus glabratus*-specific PCR.

The PCR products were ran out on a 1.5% agarose gel in 0.5 × TBE. Order of loading: GeneRuler 100 bp DNA Ladder (lanes 1 and 7), PCR pool 1 (lane 2), PCR pool 2 (lane 3), PCR pool 3 (lane 4), PCR pool 3 spiked with *X. glabratus* DNA (lane 5), no template control (lane 6). The composition of ambrosia beetle species in each PCR pool is provided in Table 3.

5.3 *Design of a* R. lauricola*-specific PCR assay*

There is an existing real-time PCR assay for *R. lauricola* (Dreaden *et al.* 2008), which was designed using the small subunit rRNA gene (SSU) sequence. However, this assay has subsequently been shown to be non-specific as an unrelated, non-pathogenic *Raffaelea* sp. (*Raffaelea* sp. isolate 272) was also detected (R. Ploetz personal communication, 2011). Alternatives to the SSU real-time PCR assay were therefore sought. The internal transcribed spacer (ITS) sequence of the rRNA gene is an unsuitable target for a PCR assay for *Raffaelea* spp. as it is very GC rich and therefore often fails to amplify. The nuclear gene β-tubulin was selected as an alternative target for diagnostic primers. When the two sequences were aligned, there was sufficient variation between the two species to design the primers RLbtubF and RLbtubR (Table 1).

The specificity of the PCR was tested with available *Raffaelea* isolates, including *Raffaelea subfusca*, *Raffaelea* sp. isolate 272 and *Raffaelea* sp. isolate DAR 34208 (Figure A1). *R. subfusca* is another symbiont of *X. glabratus* but it does not cause disease. *Raffaelea* sp. isolate DAR 34208 is transmitted by the mountain pinhole borer, *Platypus subgranosus* (Schedl), and is a pathogen of Antarctic Beech *Nothofagus cunninghamii* (Hook.f.); this is one of the few Australian native *Raffaelea* species that is available in culture collections. *R. lauricola* was positive in this assay while all other species tested negative, as well healthy avocado, demonstrating the specificity of the test.

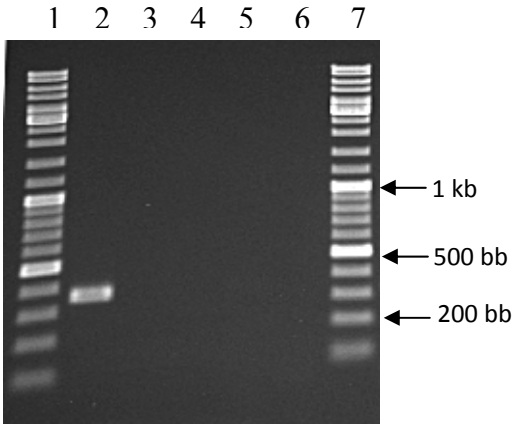

Figure 3. Specific detection of *Raffaelea lauricola* by PCR

The PCR products were ran out on a 1.5% agarose gel in 0.5 × TBE. Order of loading: GeneRuler 100 bp DNA Ladder (lanes 1 and 7), *R. lauricola* (lane 2), *Raffaelea* sp. isolate 272 (lane 3), *Raffaelea subfusca* (lane 4), *Raffaelea* sp. isolate DAR 34208 (lane 5), no template control (lane 6).

5.4 *Investigations into the cause of a laurel wilt-like disease in Queensland*

In 2010, mature avocado cv. 'Hass' trees displaying localized canopy wilt followed by branch dieback were observed on a property on the Sunshine Coast. As is characteristic of laurel wilt disease, the wilted brown leaves had not abscised. Beetle entry holes (2 mm ø) were found in the branches, mainly around the branch junction. In early infestations, there were occasionally perseitol deposits and water soaking associated with the beetle entry hole. Following molecular analysis, the beetles were identified as a new species with close affinity to *Euwallacea fornicatus* (Coleoptera: Curculionidae, Scolytinae, Xyleborina). Extensive networks of beetle galleries were present throughout the branches, and there were discoloured sections of wood surrounding these galleries. The beetles appeared selective in the branches that they chose for creation of their galleries, selecting only branches between 1.5 and 3 cm in diameter. No tree mortality was observed, but there was occasionally significant canopy thinning.

In 2011, a number of diseased avocado cv. 'Shepard' trees were observed on a property near Tolga on the Atherton Tablelands, which had similar symptoms to the aforementioned trees on the Sunshine Coast. Brown exudates and occasionally perseitol deposits were visible on the trunk of the trees, and when the bark was removed, a large number of beetle entry holes and galleries were evident (Fig. 4). The trees were infested with two ambrosia beetles, *E.* sp. aff. *fornicatus*, and a species identified in the genus *Microperus* (Coleoptera: Curculionidae, Scolytinae, Xyleborina).

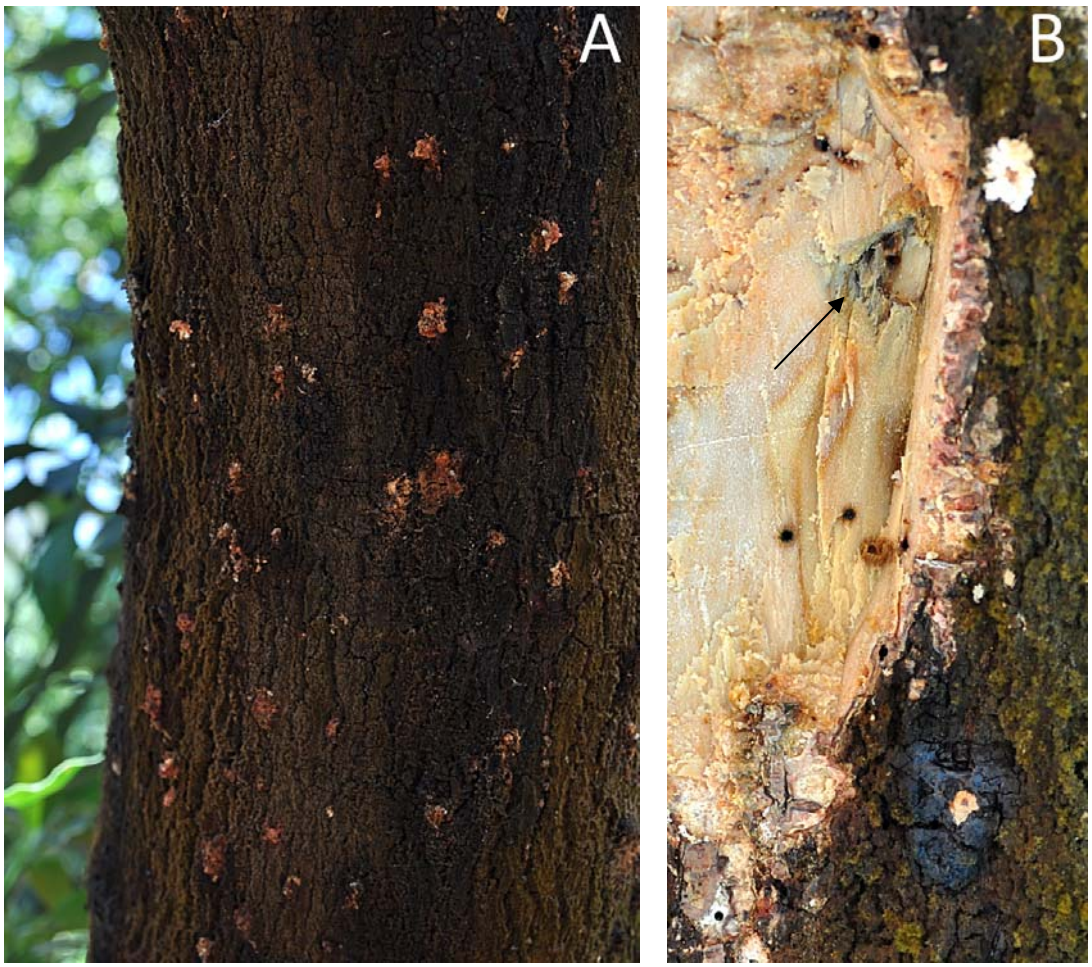

Figure 4. Damage associated with beetle activity in north Queensland.

(A) Brown exudate on trunk of mature 'Shepard' avocado due to beetle boring. (B) Beetle holes and beginning of tissue discolouration (indicated with arrow).

5.5 *Isolation of fungi from beetles and diseased trees in Queensland and satisfaction of Koch's Postulates*

A *Fusarium* species was isolated from the discoloured wood, the beetle galleries, and from the head of *E.* sp. aff. *fornicatus* from the Sunshine Coast. On the non-selective media it was the predominant fungus recovered from the beetle. An isolate was lodged with the Queensland Herbarium (BRIP 54602a). Molecular analysis showed that it is a new species (Kasson *et al*., unpublished), within a newly defined monophyletic group, designated the Ambrosia *Fusarium* Clade (AFC). From the *Microperus* sp. beetle, the predominant fungus isolated was identified by ITS sequencing as a strain of *Bionectria ochroleuca*.

To determine whether the fungal isolates that were recovered were pathogenic in nature, inoculations of healthy avocado plants were done. Throughout the experiment there were no external symptoms on any of the plants. Perseitol deposits did form around the injection hole but these were also present in control plants, suggesting they were a response to the mechanical damage. Dissection of the stems showed dark lesions extending from the inoculation site (Figure 5). There was some light staining of the tissue directly above and below the drill hole in the control plants, but no fungus was recovered from these sites, and was discounted for the lesion extension measurements.

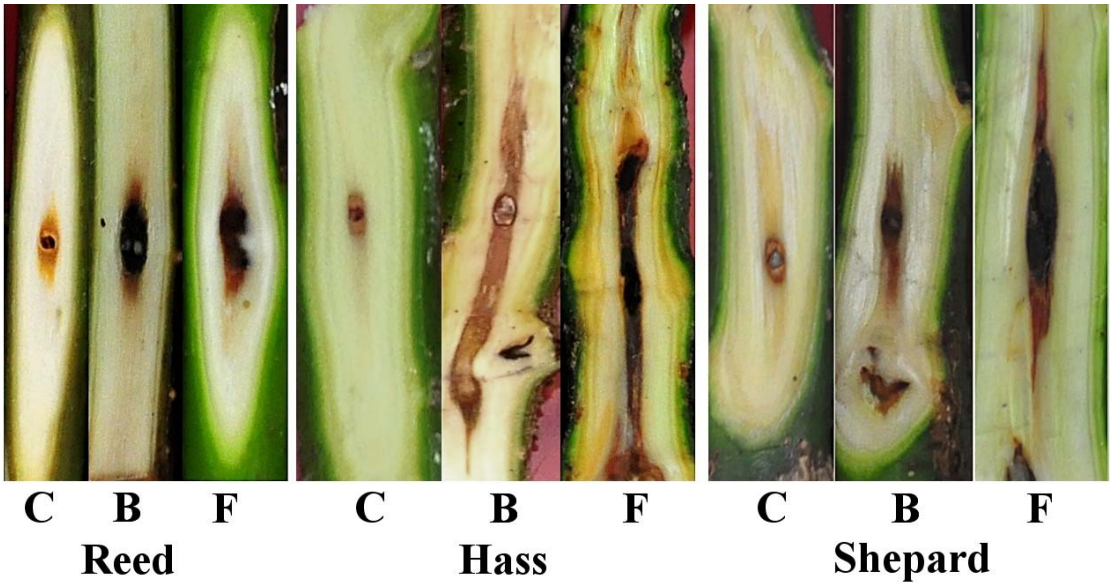

Figure 5. Examples of lesions from pathogenicity testing from the three tested cultivars. C= Sterile Water Control; B= Infected with *Bionectria*; F = Infected with *Fusarium*

Fungal cultures of *Fusarium* and *Bionectria* were re-isolated from the cankers, and were confirmed to be identical to the original isolates, thus completing Koch's postulates. The average lesion extension for each of pathogens is listed in Table 5.

Table 5. Average extension of the lesion in avocado cultivars in millimetres (std dev)

Cultivar	Control	*Bionectria*	*Fusarium*
Hass	0 0	25.40 17.38	13.08 9.98
Reed	0 0	4.92 1.09	6.58 1.18
Shepard	0 0	6.98 0.39	9.18 2.80

5.6 *Beetle surveys*

There were 396 ambrosia beetles collected in the traps (Table 6), with the majority caught with the ethanol lure. Four species were only caught with the manuka oil lure, namely *Wallacellus similis*, *Hypothenemus* sp., *Euwallacea* sp. aff. *fornicatus,* and an unidentified species. *Ambrosiodmus latecompressus* was caught in large numbers with both ethanol and manuka oil, though 77.5 % of the catch was on the manuka oil traps. Only four ambrosia beetles were caught on the unbaited traps.

Table 6. Ambrosia beetles caught in traps around avocado orchards by different lures in spring 2011

		Lure Type			
Key #	**Species Name**	**EtOH**	**Manuka**	**Unbaited**	**Totals**
1	*Ambrosiodmus latecompressus*	25	111	1	137
3	*Xyloborinus artelineatus*	12	1	0	13
4	*Diuncus adossuarius*	21	0	0	21
5	*Microperus eucalypticus*	43	1	0	44
6	*Xyleborinus saxesenii*	30	0	0	30
7	*Xylosandrus morigerus*	38	0	0	38
8	*Hypocryphalus mangiferae*	1	0	0	1
9	*Platypus omnivorus*	1	0	0	1
10	Unidentified	4	17	0	21
11	*Wallacellus similis*	0	1	0	1
12	*Hypothenemus sp*	0	1	0	1
13	Unidentified	4	0	0	4
14	Unidentified	1	0	0	1
15	Unidentified	6	0	0	6
17	*Xylopsocus gibbicollis**	30	1	1	32
18	Unidentified	2	0	0	2
21	Unidentified	3	0	0	3
22	Unidentified	0	1	0	1
23	*Xylosandrus crassiusculus*	2	0	0	2
24	*Euwallacea fornicatus*	0	1	0	1
25	Unidentified	1	0	0	1
26	Unidentified	0	0	1	1
27	*Cyclorhipidion pitogenes*	0	2	1	3
29	*Xylosandrus dicolor*	35	0	0	35
30	Unidentified	1	0	0	1
31	Unidentified	4	0	0	4
32	*Xyleborus perforans*	2	0	0	2
33	Unidentified	1	0	0	1
35	Unidentified	1	0	0	1
43	Unidentified	1	0	0	1
44	Unidentified	1	0	0	1
45	Unidentified	1	0	0	1
46	*Amasa truncata*	1	0	0	1
47	Unidentified	1	0	0	1
48	Unidentified	2	0	0	2
49	Unidentified	12	0	0	12

* *Xylopsocus gibbicollis* (common auger beetle) is not an ambrosia beetle, but was included for environmental sequencing analysis.

As morphological identification is a labour intensive process, only the most populous species were identified. The majority of beetles were trapped in Mount Tamborine region, possibly due to more favourable weather conditions for ambrosia beetles.

5.7 Environmental Barcoding

Next generation sequencing was used as a tool to investigate the fungal diversity associated with ambrosia beetles around avocado orchards. The ITS barcode produced an average of 2970 sequences over the 42 samples. There were a lot of common environmental fungi sequences (Penicillium Xylaria curta), but a number of plant pathogens, such as *Mycosphaerella* and *Fusarium*. There were 24 different species of *Fusarium* present over 23 samples (Figure 5 - *Nectriaceae*).

There are currently not enough sequences present in public databases to get proper analysis of the TEF barcode.

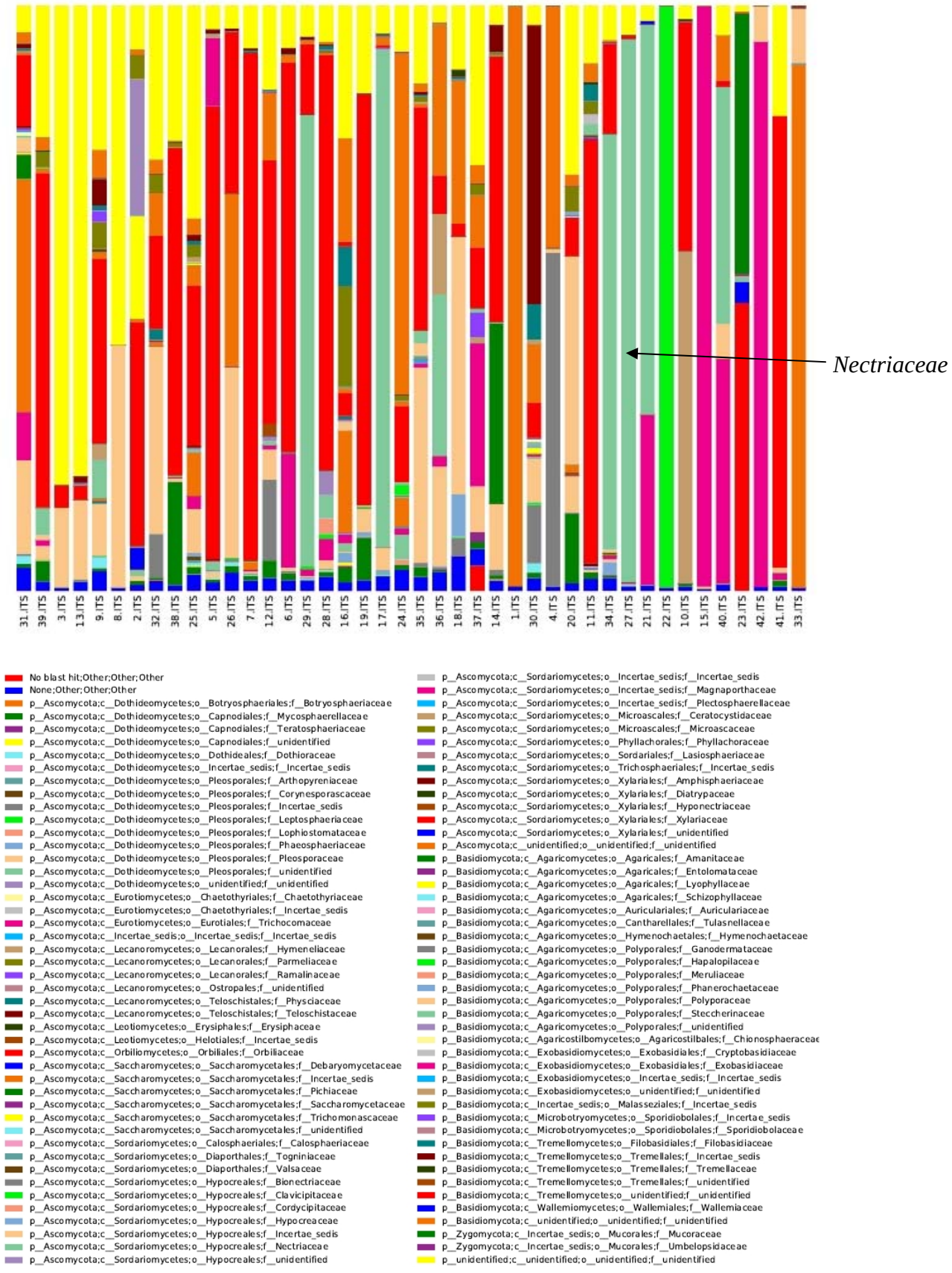

Figure 5. Summary of taxa plot at the species level for the ITS barcoding region.

6. Discussion

Until about 20 years ago, ambrosia beetles and their fungal symbionts were considered little more than a scientific curiosity, although this perception has dramatically changed in recent years with the emergence of some serious plant diseases that are directly attributable to them (Hulcr and Dunn 2011). Principal among these diseases is laurel wilt, which is ravaging native redbay forests in south-eastern USA (Shields *et al.* 2011) and poses a very serious and immediate threat to the avocado industry in Miami-Dade County, Florida.

There have been no records of *X. glabratus* from Australia and the surveys conducted in this project support this pest-free status. *X. glabratus* is not attracted to lures such as ethanol, which have routinely been used for forestry surveillance in Australia in the past, thus surveys for *X. glabratus* in particular have been suboptimal. However, sticky traps with a Manuka oil lure, a strong attractant to *X. glabratus*, were used during the course of this project and no *X. glabratus* were trapped.

X. glabratus is thought to have entered the USA in wooden packing material used to transport shipping cargo (Haack 2006). The same entry pathway exists for Australia, and given the greater proximity of Australia to Asia and the strong trade links with countries such as Japan and Taiwan, where *X. glabratus* is indigenous, it is perhaps surprising that there has yet to be an incursion of this beetle species. This may just reflect good fortune or alternatively, the conditions for establishment of *X. glabratus* in Australia may be far less favourable. In retrospect, the conditions in the south-eastern states of the USA were ideal for the establishment of *X. glabratus* because of the fact that a very attractive host tree, *Persea borbonia*, is a dominant component of the coastal vegetation of this region, including areas surrounding the major ports (Fraedrich *et al.* 2007; Menges *et al.* 1993). In contrast, hard sclerophyll forest dominated by eucalypts is the major vegetation type on the coastal plains of eastern Australia. There are native plant species from the laurel family in Australia, including those in the genera *Litsea* and *Neolitsea*, but these species are exclusively distributed in rainforests, which are mainly located on the Great Dividing Range, far from the coastal ports. Furthermore, nowhere are native Lauraceae dominant components of the vegetation. However, Camphor Laurel (*Cinnamomum camphora*) is one of the most important weed species in subtropical Australia (Batianoff and Butler 2003) and this species could possibly facilitate the establishment of *X. glabratus*.

Ambrosia beetle identification using morphological features requires great skill and experience and there are few people in Australia that could be called upon to do this task. For example, the ambrosia beetle taxonomy expert in Queensland retired more than two years ago. DNA barcoding offers an attractive option to assist with identifications, as the molecular skills needed to undertake this test are much more widely available. An output of this project has been the development of a diagnostic PCR assay for *X. glabratus*, which is based on the insect DNA barcode locus (COI gene). A positive result using this assay would indicate the presence of *X. glabratus*, and the result could be confirmed by sequencing of the amplicon and pairwise sequence comparison with specimens from the USA. Detailed protocols for running this assay have been compiled in the diagnostic manual that has been uploaded to the Plant Biosecurity Toolbox.

Evidence was obtained that at least two beetle species, *E.* sp. aff. *fornicatus* and *Microperus* sp., were reproducing in avocado trees in Queensland, and these trees had disease symptoms. An association between a particular ambrosia beetle species and disease symptoms does not necessarily indicate a causal relationship, as it is possible that the beetles had been attracted to trees with a pre-existing disease from some other cause. However, we were able to demonstrate that the fungal symbionts isolated from these beetle species were capable of causing disease symptoms when inoculated to healthy avocado plants, and that the fungi were recoverable from diseased tissue, thus satisfying Koch's postulates of pathogenicity. Of particular concern is *E.* sp. aff. *fornicatus*, which is also recorded from Israel and California (Eskalen *et al.* 2012; Mendel *et al.* 2012) and it is regarded a significant threat to avocado production in these regions.

Finally, we trialled an environmental barcoding strategy to investigate the diversity of fungi associated with ambrosia beetles in Queensland. Again, these results provided evidence of absence of the laurel wilt pathogen, *R. lauricola*. The majority of fungal species in the world are undescribed and most of those that are named do not have reference DNA barcodes. Thus, it was not possible to assign precise names to the majority of barcode sequences that were amplified from the beetles. However, of interest from a plant pathology perspective was the large number of *Fusarium* spp. that were PCR amplified, suggesting that many more diseases could be being caused by ambrosia beetles.

6. Technology Transfer

A diagnostic manual for laurel wilt disease has been produced and uploaded to the Biosecurity Toolbox Website:

http://old.padil.gov.au/pbt/index.php?q=node/46&pbtID=302.

This manual has been submitted to the Subcommittee on Plant Health Diagnostic Standards (SPHDS) for accreditation as the Australian diagnostic standard. In this diagnostic manual, detailed descriptions of the aetiology and epidemiology of laurel wilt disease and methods to identify *R. lauricola* and *X. glabratus* are provided.

Diagnostic images of laurel wilt disease have also been uploaded to the PADIL Website:

http://www.padil.gov.au/pests-and-diseases/Pest/Main/141003

The genetic characterization of the new *Fusarium* sp. from *Euwallacea* sp. aff. *fornicatus* from Australia is being published in the following paper:

Kasson MT, O'Donnell K, et al. (2013) An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts avocado and other plant hosts. Fungal Genetics and Biology (submitted).

Results of this project were also presented at the World Avocado Congress 2011, Cairns, Australia. 5 – 9 September 2011:

http://worldavocadocongress2011.com/?PageID=35

7. Recommendations

The recommendations from this project are as follows:

(i) This project should be viewed as only part of an ongoing biosecurity capacity building process for the Australian avocado industry. The diagnostic procedures that have been produced by us address one of a long list of high risk biosecurity threats, as outlined in the *Industry Biosecurity Plan for the Avocado Industry Version 2 September 2011*. Diagnostic manuals still need to be produced for *Sphaceloma perseae*, *Phytophthora kernoviae* and *P. mengei*, among others. Furthermore, it is necessary to regularly revise existing diagnostic manuals, as technologies change and new information becomes available. For example, in the period since the experimental component of this project finished, a new species of *Raffaelea* has been identified in a Californian avocado with laurel wilt-like disease symptoms (Eskalen and McDonald 2012). It is recommended that diagnostic manuals be updated biennially.

(ii) The emergence of laurel wilt in Florida has heightened awareness among avocado pathologists of plant diseases that are vectored by ambrosia beetles. During the course of this project, a new disease of avocado caused by *Fusarium ambrosium* and transmitted by *Euwallacea fornicatus* was identified in Australia. This disease possibly poses an even greater and more immediate threat to the Australian avocado industry than laurel wilt as it is already here and *E. fornicatus* is more of a habitat generalist than *X. glabratus*. In the past, symptoms of *F. ambrosium* infection could easily have been confused with those caused by *Verticillium dahliae* or *Phytophthora cinnamomi*. It is recommended that more research be done on *E. fornicatus*/*F. ambrosium* in order to better quantify the impact of these organisms on avocado production in Australia, which will inform decisions as to whether control measures are needed.

(iii) Taxonomic expertise in entomology and mycology is becoming progressively scarcer in Australia, which will compromise the nation's ability to manage biosecurity issues. One solution to this taxonomic 'brain drain' is to use DNA barcoding for pest and pathogen identifications, although the success of such a technique depends on the availability of an accurate and comprehensive DNA barcode database. It is recommended that work be done to fill in any gaps that may exist in reference DNA barcodes for pests and pathogens of avocado both here in Australia and overseas.

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