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Title: Members of the *Euwallacea fornicatus* species complex exhibit promiscuous mutualism with ambrosia fungi in Taiwan

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Abstract

Carrillo, J.D., Rugman-Jones, PF., Husein, D., Stajich, J.E., Kasson, MT., Carrillo, D., Stouthamer, R., and Eskalen, A. 2019. Members of the *Euwallacea fornicatus* species complex exhibit promiscuous mutualism with ambrosia fungi in Taiwan

A number of ambrosia beetles have come to prominence in recent years because of the damage they inflict on a variety of trees within invaded habitats across the globe. Ambrosia beetles rely on symbiotic microorganisms, mainly fungi, as a dedicated food source and carry those microorganisms around with them within specialized organs termed mycangia. Investigation of members of the *Euwallacea fornicatus* species complex and their fungal symbionts in Taiwan revealed promiscuous symbioses with ambrosial Fusaria clade (AFC) members, *Graphium* spp., and *Paracremonium* spp. based on co-phylogenetic analyses. For AFC members, a novel diagnostic PCR assay targeting mating type genes *MAT1-1-1* and *MAT1-2-1* was developed and validated by amplicon size and sequencing. Mating types screening of AFC members revealed the isolates screened are all heterothallic (self-sterile), with both *MAT* types represented and recovered from fungi vectored by *E. fornicatus*, *E. kuroshio*, and *E. whitfordiodendrus* in Taiwan. Members of the *Euwallacea fornicatus* species complex and the variety of ambrosia

fungi they utilize further confirms that their relationship with fungi are more likely promiscuous in native areas, as opposed to strictly obligate to a specific combination of fungi as observed in invaded areas.

1. Introduction

Bark and ambrosia beetles are well-known forest insects which have the potential to cause economic damage to trees and timber (Beaver 1989; Hulcr and Stelinski 2017). Interest in ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) has increased in recent years because of the damage they can cause when they invade a new area (Carrillo et al. 2014; Eskalen et al. 2013; Mendel et al. 2012; O'Donnell et al. 2016; Hulcr and Stelinski 2017). Although the vast majority of ambrosia beetles have minimal effect on forest health and ecosystem function, there are a few ambrosia beetle species that have become serious pests in invaded areas such as *Xyleborus glabratus, Xylosandrus* spp., *Euwallacea* spp., *Trypodendron* spp., and *Gnathotrichus* spp. (Eskalen et al. 2013; Carrillo et al. 2014; Hulcr and Stelinski 2017; O'Donnell et al. 2016). Ambrosia beetles rely on symbiotic microorganisms, mainly fungi, as a dedicated food source and carry those microorganisms around with them within specialized organs termed mycangia (Batra 1963). The beetles tunnel into trees and cultivate their symbiotic organisms on nutritionally poor xylem tissue, forming "fungal gardens" inside colonized hosts (Beaver 1989). However, in addition to providing food for the beetles, these fungi can be pathogenic to the plants they colonize, which can lead to tree dieback or mortality as a result of inoculation with a particularly virulent fungus or by mass inoculation of fungal pathogens by the pests (Hulcr and Stelinski 2017). For example, the invasive red bay ambrosia beetle *Xyleborus glabratus* vectors a virulent primary symbiont, *Raffalea lauricola*, and has killed over a half-billion trees in the decade following its introduction into southeastern USA (Hughes, 2013). *Euwallacea fornicatus* Eichoff and close relatives represents another group of ambrosia beetles that may prove to be equally destructive (Eskalen et al. 2013; O'Donnell et al. 2016).

Euwallacea fornicatus sensu lato is in fact a complex of several morphologically very similar species (O'Donnell et al. 2015; Stouthamer et al. 2017; Gomez et al. 2018). Members of this complex have recently invaded areas of the United States (including California, Florida, Hawaii, and much of the mid-Atlantic and Southeastern U.S. (CABI 2015; Cognato et al. 2015; Eskalen et al. 2013; Na et al. 2017; O'Donnell et al. 2015; Rabaglia et al. 2006) and other parts of the world including Australia, Costa Rica, Guatemala, Israel, Panama, and South Africa, (CABI 2015; Garcia et al. 2016; Paap et al. 2018; Stouthamer et al. 2017). *Euwallacea fornicatus* sensu stricto

(Gomez et al. 2018), commonly known as the tea shot hole borer (TSHB), is associated with the fungal symbiont *Fusarium ambrosium* Gadd & Loos and is a serious pest of tea (*Camellia sinensis*) in its native India and Sri Lanka (Danthanarayana 1968). However, TSHB has recently established in Florida, USA, where it has become a pest of avocado trees (Carrillo et al. 2016). Similarly, in California, USA (Eskalen et al. 2013), Israel (Mendel et al. 2012), and South Africa (Paap et al. 2018), *E. whitfordiodendrus* Schedl, known commonly as polyphagous shot hole borer (PSHB) is widespread following its inadvertent introduction and is a pest of avocado in each of those three areas. Rather than *F. ambrosium*, these invasive populations of PSHB are associated with a trio of symbiotic fungi; *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, *Graphium euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen, and *Paracremonium pembeum* S.C. Lynch & Eskalen. PSHB appears to have a vast host range, and in addition to avocado, it is also a significant threat to trees in urban landscapes and natural habitats (Eskalen et al. 2013). One survey in California has shown that a third of native California sycamore trees (*Platanus racemosa*) in Orange County public parks are infested with PSHB, and since 2014, the beetle has caused the removal of 1262 trees, at a cost of approximately 4 million USD (OC Parks, 2017). A third species, *E. kuroshio* Gomez and Hulcr, known as the Kuroshio shot hole borer (KSHB), has also established in California (Na et al. 2018; Stouthamer et al. 2017), and is again associated with a seemingly unique set of fungal symbionts; *Fusarium kuroshium* F. Na, J. D. Carrillo & A. Eskalen and *Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen. KSHB is thought to have a similar host range to PSHB and represents a similar threat to urban and natural environments (Boland 2016).

It has been traditionally assumed that ambrosia beetle species are intimately associated with a single dominant symbiotic fungus (Kostocvik et al. 2015). However, it has been shown that they may feed on more than one species in fungal galleries (Batra 1966; Lynch et al. 2016) and in PSHB, the proportion of fungal species devoured/harbored by individuals shifts during beetle development and maturation (Freeman et al. 2016). Lateral transfer of fungal symbionts has also been reported between other sympatric ambrosia beetle species (Kostocvik et al. 2015), which may be facilitated by the beetles colonizing the same host (Carrillo et al. 2014). Current knowledge of the fungal symbionts carried within the mandibular mycangia of members of the *E. fornicatus* species complex has been based on the examination of invasive, and hence isolated, populations (Eskalen et al. 2013; Kasson et al. 2013; Mendel et al. 2012; O'Donnell et al. 2015). As a result, it has been suggested that PSHB and TSHB are strictly obligate with respect to their *F. euwallaceae* and *F. ambrosium* mutualists, respectively (Freeman et al.

2013). Promiscuous symbiosis has been reported in many other ambrosia beetle species in invaded areas such as Florida (Carrillo et al. 2014; Kostocvik et al. 2015), but the occurrence of this phenomena within the *E. fornicatus* species complex has limited investigation in native and invaded habitats.

Fusarium spp. associated with *Euwallacea* spp. are thought to be the main adult and larval food source, or primary symbionts, and have been collectively referred to as the ambrosia Fusaria clade (AFC), with novel species being numbered (Kasson et al. 2013; O'Donnell et al. 2015). TSHB, PSHB, and KSHB are all native to south and southeast Asia (Beaver, 1989; Hulcr and Stelinski 2016; Stouthamer et al. 2017) with recent studies finding additional novel species of AFC members within clade "B" (Kasson et al. 2013) from native habitats, such as Taiwan (Na et al. 2017). Other fungi involved in these mutualisms, such as *Graphium* spp. and *P. pembeum*, have been described associated with invasive *Euwallacea* spp. (Lynch et al. 2016; Na et al. 2018) with *Graphium* spp. thought to have a function in supporting larval development (Freeman et al. 2015). Although involved in the symbiosis from invaders, *Graphium* spp. and *Paracremonium* spp. have not been investigated in native Southeast Asia regions. Sampling and identification of *Euwallacea* spp. at the cytochrome oxidase I (COI) locus in Taiwan found multiple haplogroups of *E. fornicatus* species complex members present in the region (Stouthamer et al. 2017). Investigation into beetle-fungi relationships for fungal symbionts including AFC members, *Graphium* spp., and *Paracremonium* spp., in native areas can help identify the specific or non-specific association of the beetles and their ambrosia fungi which has implications in invaded areas with multiple beetle and fungal species present, such as California and Florida.

The mycangial symbionts of ambrosia beetles had long been assumed to be strictly asexual, but recent discoveries have overturned these long-held assumptions and opened the door for broader investigations across independently evolved fungal clades (Mayers et al. 2017). Sexual recombination occurrence within AFC fusaria has not been observed to date, but is well described in other plant pathogenic *Fusarium* spp. such as the *F. graminarium* species complex (teleomorph *Gibberella zeae,* Bowden and Leslie 1999)*, F. fujikuroi* species complex (teleomorph *G. fujikuroi,* Kuhlman 1982), and *F. solani* species complex (teleomorph *Nectria haematococca*, Booth 1960), with the latter being the species complex that contains AFC fusaria. Mating type in *Fusarium* spp. is controlled by a single locus (*MAT)* with two idiomorphic alleles, termed *MAT1-1* and *MAT1-2* (Kim et al. 2012; Leslie and Summerell 2008). Fungal mating systems in *Fusarium* spp. can be homothallic (self-fertile), as seen in the *F.*

graminarium species complex where strains can carry both *MAT1-1* and *MAT1-2* idiomorphs, or heterothallic (self-sterile) as seen with strains of *F. fujikuroi* (Yun et al. 2000). Members in the *F. solani* species complex have been reported to contain species with heterothallic and homothallic members as well as species with no known sexual stage, which was initially hypothesized for the *F. ambrosium* associated with TSHB (O'Donnell 2000). Plant pathogenic fungi can recombine to combat selective pressures such as host resistance genes and fungicide application, which has been reported in *F. gramanarium* on wheat (Miedaner et al. 2001). Current identification of *MAT* in *Fusarium* spp. is done using PCR assays which specifically target conserved *MAT1-1* (α-BOX) and *MAT1-2* (HMG BOX) regions of *Fusarium* spp. to amplify mating type idiomorphs of both sexually (Steenkamp et al. 2002) and asexually reproducing species (Kerenyi et al. 2004). This has been validated on some *F. solani* species complex members but has not for AFC members. It is unknown if AFC members associated with TSHB, PSHB, and KSHB are homothallic or heterothallic nor has a reliable assay been validated by PCR amplification aimed at targeting *MAT* regions of AFC fusaria to type strains in native and invaded areas.

The objectives of this study were to investigate beetle-fungi association with *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. recovered from *Euwallacea* spp. in native habitats through identification of the beetle species haplogroup (Stouthamer et al. 2017) coupled with identification of the recovered fungal species recovered from the beetles or inhabited galleries using multi-gene phylogenetics. Of the AFC fusaria members, we aimed to develop and validate a PCR assay to investigate mating types present in the population from AFC members recovered from the *Euwallacea* spp. sampled in Taiwan as well as other known AFC species associated with invasive *Euwallacea* spp. The AFC-specific assay can be a useful tool to identify mating types among AFC members introduced and vectored by *Euwallacea* spp. in native and invaded areas throughout the world.

2. Materials and methods

2.1. Sample collection and isolation

In 2017, infested avocado (*Persea americana* Mill.) branches, mostly old pruning wounds, approximately 5 cm diameter x 25 cm length, were collected from four different locations in the Danei District of Taiwan and shipped to a BSL-2 insectary and quarantine facility at UC Riverside under USDA APHIS permit (P526P-16-03142). During shipping, the wood was stored in separate ventilated plastic bags representing each location sampled. Upon arrival at the insectary and quarantine facility, the logs were placed individually in isolated plastic chambers with mesh

ventilation to allow airflow and prevent beetles from escaping. Females were collected from the wood as they emerged from their natal galleries and a total of 130 *Euwallacea* sp. nr. *fornicatus* females were collected. Fungal isolates used in this study were obtained from the heads of female beetles similar to methods described by Lynch et al. (2016) and from wood recovered from *Euwallacea* spp. galleries in their reproductive host trees, similar to methods described by Eskalen et al. (2013).The beetles were surface sterilized by submerging in 70% ethanol and vortexed for 20 s, rinsed with sterile de-ionized water, and allowed to dry on sterile filter paper. Beetle heads were separated from the thoracic and abdominal segments under a dissection microscope, then the head segments were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles. The macerated heads were suspended in 1 ml of sterile water and 25 µl of the suspensions were pipetted onto Petri plates containing potato dextrose agar (PDA; BD Difco, Sparks, MD) amended with 0.01% (w/v) tetracycline hydrochloride (PDA-t) and spread using sterile glass L-shaped rods. Plates were incubated for two days at room temperature and single spore fungal colonies with unique morphologies were sub-cultured for further downstream identification. The remaining abdomen/thorax segments were saved for downstream beetle identification to link the fungi to the beetles they came from. In 2018, 40 infested gallery samples from avocado, with live beetles present, were excised with a sterile knife and cut into small pieces that would fit into a 2ml microcentrifuge tube. The beetles that came from the sampled wood were placed into a separate microcentrifuge tube and suspended in 70% ethanol to match the wood they came from. Because of the required suspension of beetles in ethanol during shipping, fungal symbionts were not able to be recovered from their mycangia. Both gallery samples and beetles that occupied them were shipped directly to UC Riverside under USDA-APHIS permit (USDA-APHIS AP17PPQS&T00C01). Fungal isolation from wood was performed under a bio-safety cabinet (BSC II) and wood was surface sterilized and plated onto PDA-t petri plates as well as Malachite Green Agar (MGA; Leslie and Summerell 2006) to select for *Fusarium* spp. Plates were incubated at 25°C for five days, and emerging colonies were scraped with a sterile loop, streaked out on PDA-t, and single colonies were isolated after incubation for two days at 25°C. Unique morphologies from *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. were selected for downstream identification.

2.2. DNA extraction, PCR, and phylogenetic analysis

Genomic DNA of the fungal isolates of unique morphology obtained from *Euwallacea* spp. beetles and gallery samples were extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany). Approximately 25mg of

fungal mycelium was harvested from fungal isolates (*Fusarium* spp., *Graphium* spp., and *Paracremonium* spp.) from fully colonized PDA and placed into sterile 1.5 mL microcentrifuge tubes previously loaded with 25µl of AP1 buffer (Qiagen, Hilden, Germany) then frozen at 0°C and macerated with a plastic pestle (Thermo Fisher Scientific, Pittsburgh, PA, USA). Once the tissue was macerated, the DNeasy plant mini kit (Qiagen, Hilden, Germany) manufacturer protocol was used to extract DNA from the samples. All samples were suspended in 50µl AE elution buffer and DNA concentration was quantified using a Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA).

PCR amplification of internal transcribed spacer (ITS)1-5.8S-ITS2 and translation elongation factor 1-α (TEF1-α) was done using ITS4/ITS5 (White et al., 1990) for all species tested, EF1/EF2 for *Fusarium* spp. only (O'Donnell et al. 1998), and EF1F/EF2R for *Graphium* spp. and *Paracremonium* spp. only (Jacobs et al. 2004). In addition, PCR amplification of RNA polymerase subunit I (RPB1) and RNA polymerase subunit II (RPB2) loci was performed for *Fusarium* spp. and *Paracremonium* spp. only using primers F5/R8 (RPB1-1) (O'Donnell et al. 2010), F7/G2R (RPB1-2) (O'Donnell et al. 2010), 5F2/7CR (RPB2-1) (O'Donnell et al. 2007), 7CF/11AR (RPB2-2) (O'Donnell et al. 2007). Two additional genes were sequenced for *Paracremonium* spp. including calmodulin (cal) using CAL-228F and CAL2Rd (Carbone and Kohn, 1999) and ATP citrate lyase region (acl1) with acl1-230up and acl1-1220low primers (Gräfenhan et al. 2011). It should be noted that LSU domains D1 and D2 of the LSU rDNA were previously found to be least informative (Kasson et al. 2013, O'Donnell et al. 2015) therefore were not included in this study. Each PCR reaction mixture consisted of 12.5 µl GoTaq DNA Polymerase (Promega, Madison, WI), 9.3 µl sterile DNase-free water, 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer PCR, and 2 µl of 10ng genomic DNA template to a total of 25 µl reaction mixture. PCR was performed for each primer set using published cycling parameters (Carbone and Kohn 1999; Groenewald et al., 2013; Jacobs et al. 2004; O'Donnell et al. 1998; O'Donnell et al. 2007; White et al. 1990). Amplified products were separated by gel electrophoresis in 1% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and viewed under UV light. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) then sequenced in both directions (Sanger ABI3730) at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers used for PCR. Raw sequences were assembled in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI). The species identity of each *Euwallacea* specimen was confirmed by sequencing the mitochondrial COI gene via DNA extraction and amplification protocols described by Stouthamer et

al. (2017). Specimens were subsequently diagnosed as PSHB, KSHB, or TSHB, and haplotypes were identified using BLAST searches against sequences from that study (GenBank KU726991-KU727041).

Multi-gene phylogenetic analysis was conducted to determine the genetic relatedness of *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. isolates obtained from sampled locations in Taiwan. These isolates were also compared to other members of their respective genera. Phylogenetic analysis of *Fusarium* spp. was conducted using concatenated DNA sequences at ITS, TEF1-α, RPB1, and RPB2 gene regions from thirty-two isolates of *Fusarium* spp. from Taiwan (Table 1), along with DNA sequences obtained from GenBank (Table 2) from thirty-six isolates previously used in AFC phylogenetic analysis from Kasson et al. (2013) and O'Donnell et al. (2015). Phylogenetic analysis of *Graphium* spp. was conducted using concatenated DNA sequences of ITS and TEF1-α gene regions from twelve isolates of *Graphium* spp. (Table 1) as well as twenty-eight isolates obtained from GenBank (Table 2) used in a previous analysis by Lynch et al. (2016). Phylogenetic analysis of *Paracremonium* spp. was conducted using concatenated DNA sequences of ITS, TEF1-α, RPB2-1, cmd, and acl1 gene regions from eight isolates of *Paracremonium* spp. (Table 1) as well as eleven isolates obtained from GenBank (Table 2) used in a previous analysis by Lynch et al. (2016). All respective sequences from *Fusarium* spp. *Graphium* spp., and *Paracremonium* spp. were aligned using Clustal X (Thompson et al. 1997) and concatenated after alignment. A partition file was created to indicate the range of each gene in the concatenated alignment and allow for different substitution models. The multigene phylogenies were constructed with Maximum Likelihood (ML) methods using IQ-TREE (Nguyen et al. 2015). The ModelFinder option (Kalyaanamoorthy et al., 2017) was used to determine that the best partitioning scheme with the TESTMERGE command (Chernomor et al. 2016) was TEF, and ITS+ RPB1+RPB2 for *Fusarium* spp., ITS+EF for *Graphium* spp., and ITS,TEF,RPB2,ACL,CAL for *Paracremonium* spp. identified the best substitution model for each of the defined partitions (-m MFP -nt AUTO -spp partitions.txt –bb 1000) of *Fusarium* spp., for each of the two defined partitions (-bb 1000 -m TESTNEW -q partitions.txt) of *Graphium* spp., and for five defined partitions (-m MFP -nt AUTO -spp partitions.txt –bb 1000) of *Paracremonium* spp.. The best model to use was TIM3e+I+G4 (ITS+RPB1+RPB2) and K2P+G4 (EF) for *Fusarium* spp., K2P+I (ITS+EF) for *Graphium* spp., and TNe+G4 (ITS), TNe (TEF), TIM3e+G4 (RPB2), K2P+I (acl1), HKY+F+I+G4 (cal) for *Paracremonium* spp. IQ-TREE was run with 1000 standard bootstrap analyses to generate final tree run.

2.3. Cophylogenetic analysis

Evidence for cophylogeny between *Euwallacea* spp. and their symbiotic fungi was analyzed separately for *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. Resultant IQ-TREE ML tree files for each group were directly compared to phylogenies for *Euwallacea* spp. built from sequences described in O'Donnell et al. (2015) were used as input to build distance matrices. Host-symbiont matrices were manually created using the identities of the fungi and which *Euwallacea* spp. they were recovered from and included in the cophylogenetic test using "parafit" function (Legendre et al. 2002) within the package "ape" (Paradis et al. 2004) in R version 3.4.3 (Team 2013). In parafit analysis, the distance matrices from the *Euwallacea* spp. and symbiotic ambrosia fungi were computed from the generated ML trees. Probabilities were based on 999 permutations and the correlation was considered significant at $P < 0.05$. The null hypothesis of the global test is that the associations between *Euwallacea* spp. and their symbiotic ambrosia fungi are randomly distributed on the phylogeny. The null hypothesis for individual links is *Euwallacea* spp. and symbiotic ambrosia fungi association is established at random. The datasets generated during the current study are available on a Github repository: (https://github.com/jcarr022/Taiwan_Euwallacea_Fungi)

2.4. Primer design and validation for mating type genes of AFC fusaria

Available genomes from AFC species (GenBank Accession: GenBank Accession: NHTE00000000 = *F. euwallaceae*, NKUJ00000000 = *F. kuroshium*, NIZV00000000 = *F. ambrosium*), NKCL00000000 = *Fusarium* sp. AF-3, NKCK00000000 = *F. oligoseptatum*, NKCJ00000000 = *Fusarium* sp. AF-6, NKCI00000000 = *Fusarium* sp. AF-8) were utilized to design novel primers that can identify AFC mating types (*MAT*) from both AFC clade A and B (Kasson et al. 2013; O'Donnell et al. 2015) from extracted and aligned sequences from both *MAT 1-1-1* and *MAT 1-2-1*. Conserved regions both *MAT 1-1-1* and *MAT 1-2-1* genes were selected and melting temperatures and secondary structures of generated oligonucleotides were evaluated using Primer3 (v. 0.4.0, Koressaar and Remm, 2007; Untergasser et al. 2012). Candidate oligonucleotide sequences were also checked for specificity by using Fasta36 (Pearson 2016) against AFC genomes using the -fasta36 command. The *MAT* regions used for AFC_MAT1 primers was a partial region from the *MAT 1-1-1* gene, while AFC_MAT2 primers were designed around a partial region of the *MAT 1-2-1* gene (Table 4). The primers were optimized using a gradient. The PCR reactions were carried out in 25µl reactions using GoTaq Green Master Mix (Promega, Madison, WI) 250nm of each primer set, and 1µl DNA template (at 10ng/ul) or nuclease free water (Thermo Fisher Scientific, Pittsburgh, PA, USA) for non-

treated controls (NTC). The optimal conditions for all primer sets was determined to be a three step protocol: 95°C for 2 m followed by 30 cycles of denaturation at 95°C for 30 s followed by annealing at 60°C for 30 s followed by extension at 72°C for 30 s and a final extension at 72°C for 5 m by using conventional PCR thermal gradient option with a MyCycler® (Bio-Rad, Hercules, CA, USA). End-point analysis of amplified products was done by agarose gel electrophoresis in 1% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and visualized under UV light. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) then sequenced in both directions at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers used for PCR. Raw sequences were assembled in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI). Products were validated by using basic local alignment search tool (BLAST, Altschul et al. 1990) against GenBank accessions from closely related *Fusarium* spp. and submitted to GenBank (Table 1).

3. Results

3.1. Phylogenetic diversity of fungal symbionts associated with *Euwallacea* spp. in Taiwan

For phylogenetic trees using *Fusarium* spp., sequences of *Fusarium neocosmosporiellum* (NRRL 22468, NRRL 43467) and *F. lichenicola* (NRRL 32434) were used as outgroups for rooting the *Fusarium* tree (Fig. 1) based on prior analyses of AFC members (Kasson et al. 2013; O'Donnell et al. 2015, Na et al. 2017). Multi-locus phylogenetic analysis performed on four loci (ITS, TEF1-α, RPB1, and RPB2) from *Fusarium* isolates tested in this study indicate *Fusarium* spp. recovered from *Euwallacea* spp. in Taiwan represent AFC species ([AF-13]-[AF-16]) found from Taiwan isolates previously (Na et al. 2017) and reside in AFC species major clade B (Kasson et al. 2013; O'Donnell et al. 2015). Interestingly, the AFC species from Taiwan are paraphyletic, with AF-16 forming a monophyletic clade that is sister to AF-[2-4;12-15]. With more isolates representing AF-16 compared to Na et al. (2017), two additional sister clades were resolved within AF-16, including AF-17 and AF-18 (Fig. 1).

Sequences from previous phylogenetic analysis of *Graphium* spp. (Lynch et al. 2016) were used to build a maximum likelihood phylogenetic tree through multi-locus phylogenetic analysis using two informative loci, ITS and TEF1-α including *Graphium* spp. recovered from *Euwallacea* spp. in Taiwan (Fig 2, Supplemental Fig. S1). Two isolates (UCR 5548, 5528) recovered from PSHB in Taiwan were found to form a well-supported clade (95% bootstrap support) within the *G. euwallaceae* clade, while all other isolates recovered from PSHB in Taiwan resolving within the *G. kuroshium* clade (Fig. 2) which was initially described when recovered from KSHB in

California (Na et al. 2017). *Graphium* spp. recovered from TSHB in Florida formed their own distinct clade (88 % bootstrap support) sister to the *G. euwallaceae* clade and the *G. carboniaruim* clade. In addition to *Graphium* spp., *Paracremonium* spp. were also found in association with *Euwallacea* spp. in Taiwan. *Paracremonium* isolates grouped with isolates of *Paracremonium* sp. I from Vietnam, which were initially described by Lynch et al. (2016) (Fig. 3). *Paracremonium* spp. isolates recovered from an invasive TSHB population in Florida also fell into this group but those obtained from invasive PSHB and KSHB populations in California were identified as a different species, *P. pembeum*.

3.2. Fungal symbiont promiscuity in *Euwallacea* spp. in Taiwan

No evidence for symbiont fidelity was found between AFC and *Euwallacea* species in Taiwan. AFC Fusaria ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results ($P = 0.72$) as well as individual links ($P > 0.05$). However, the composition of beetle species emerging from the infested avocado logs from Taiwan was heavily biased toward PSHB (corresponding to haplotypes H22 and H38 in Stouthamer et al. 2017; Table 3). Fungi recovered from female mycangia and individual galleries the sampled female was located consisted of seven AFC species identified in Taiwan: AF-18 was isolated from all three species (and four haplotypes), PSHB (H22 & H38), TSHB, and KSHB; AF-16 and AF-17 were isolated from both PSHB (H22 & H38) and TSHB while AF-13 and AF-14 were isolated from both PSHB (H38) and TSHB. Indeed, AF-15 was the only *Fusarium* species recovered from just a single *Euwallacea* species/haplotype (PSHB H38). *F. kuroshium* was recovered from PSHB (H38) from our sampling efforts in 2017 and 2018 when it had been previously described associated with KSHB in California (Fig. 1).

Four species of *Graphium* were recovered from the mycangia of newly emerged *Euwallacea* spp. females from sampled logs (Fig. 2, Supplemental Fig. S1). *Graphium kuroshium* was isolated from both PSHB (H38) and KSHB (H20), and two further species that were closely related to *G. kuroshium* were also isolated only from PSHB (H38). A species related to *G. euwallaceae* was also isolated only from PSHB (H38). A unique *Graphium* species was isolated from the invasive TSHB population in Florida, but in Taiwan, no *Graphium* species were isolated from TSHB (H8) (Fig. 2, Supplemental Fig. S1) Cophylogenetic parafit analysis of the *Euwallacea* spp. and *Graphium* spp. ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results ($P = 0.14$) but individual link association of *Graphium* sp. I from *E. validus* was found to be non-random

(*P*= 0.03) while the other individual link associations *were* random (*P* >0.05). PSHB (H38) from Taiwan were found to be associated with *G. kuroshium*, a species clade closely related to *G. kuroshium*, as well as a species sister to *G. euwallaceae*. A single *Paracremonium* species (*Paracremonium* sp. I; Lynch et al. 2016) was detected in Taiwan which was isolated from both PSHB (H38) and TSHB (H8) (Fig. 3). The same species was isolated from the invasive population of TSHB (H8) in Florida. Cophylogenetic parafit analysis of the *Euwallacea* spp. and *Paracremonium* spp. ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results (*P* = 0.18) as well as individual links (*P* >0.05). *Paracremonium pembeum* (carried by invasive PSHB populations) was not detected in Taiwan but was recovered from the invasive KSHB population in California.

3.3. PCR assay for determining AFC mating type

The degenerate primers (Table 4) for the novel PCR assay for screening *MAT* type targeting AFC members was found to produce products in all AFC isolates tested including AF [1-2]; AF-[4-6]; AF-[8-18] (Table 5). AFC members were found to be heterothallic with all isolates tested either showing product for *MAT1-1-1* or *MAT1-2-1*. The invasive AFC species *F. euwallaceae* [AF-2] and *F. kuroshium* [AF-12] appear to be both MAT1-1 while *Fusarium* spp. [AF-6] in Florida has both mating types present in the population. Product size for *MAT1-1-1* was approximately 550 bp for AF-2 (*F. euwallaceae)* and AF-12 (*F. kuroshium*) and 600 bp for Taiwan isolates, while *MAT1-2-1* for Taiwan isolates was 800 bp (Fig. 4). Sequences obtained from isolates validated *MAT* products as *MAT1-1-1* or *MAT1-2-1* from *in silico* BLAST results from closely related species (data not shown). Within promiscuous AFC species (AF-[13,14,16-18]) isolates included in the phylogenetic study, *Euwallacea* spp. in Taiwan were found to vector different AFC species while also vectoring both mating types *within* the AFC species (Fig. 1). Within sampled regions in Taiwan, both mating types were represented among the sampled regions (Table 3).

4. Discussion

Investigation into the association of members from the *Euwallacea fornicatus* species complex (PSHB, TSHB, and KSHB) with their symbiotic fungi in Taiwan revealed evidence for non-specific association with AFC members, *Graphium* spp., as well as *Paracremonium* spp. from co-phylogenetic analyses (Figs 1,2,3, Supplemental Fig. S1) on samples recovered from female mycangia and galleries. *Euwallacea fornicatus* species complex

members in invaded areas have been initially reported to be associated exclusively with specific AFC members (Freeman et al. 2013; O'Donnell et al. 2015) as well as *Graphium* spp. and *Paracremonium* spp. (Lynch et al 2016; Na et al. 2017), where in native habitats like Southeast Asia, we recovered a variety of AFC members, *Graphium* spp., and *Paracremonium* spp. across multiple beetle haplogroups within the *E. fornicatus* species complex. In addition, AFC members were found to be heterothallic (Fig. 4); with both *MAT* types represented and recovered from *E. fornicatus* species complex members inhabiting host wood in the indigenous region (Table 3). The diagnostic assay targeting *MAT* types may be a useful diagnostic tool to type invasive AFC members associated with *E. fornicatus* species complex members to investigate if both idiomorphs are present in invaded areas and if mating is occurring in said areas. The relationship of members from the *E. fornicatus* species complex with the variety of ambrosia fungi that was recovered from female mycangia and isolated galleries is likely promiscuous, as opposed to strictly obligate to specific fungal species as currently described in invaded areas.

The AFC species recovered from *Euwallacea* spp. samples in this study represent the same species clades described from previously reported Taiwan AFC isolates (Na et al. 2017), which found new AFC species AF-13 to AF-16. Our sampling efforts from this study discovered more isolates within the AF-16 clade which diverge to 3 strongly supported clades which we are terming AF-16 (98% bootstrap), AF-17 (90% bootstrap), and AF-18 (98% bootstrap) (Fig. 1) determined using a multi-gene phylogeny. Interestingly, UCR6408 was identical to *F. kuroshium* (AF-12) associated with KSHB in California, however it was recovered from PSHB mycangia in this study. All of the *Graphium* spp. recovered from *Euwallacea* spp. in this study were found to be closely related to either *G. euwallaceae* or *G. kuroshium* (Figs. 2, 3). Two isolates UCR5519 and UCR5549 were found to be identical to *G. kuroshium* associated with KSHB in California, but again was recovered from PSHB mycangia as opposed to KSHB in California. The finding of *F. kuroshium* and *G. kuroshium* corroborates the findings of Stouthamer et al. 2017 that Taiwan is a likely origin of invasive KSHB-FD in California.

Paracremonium spp. found from isolated beetle infested galleries in Taiwan and Florida did not clade with *P. pembeum* associated with PSHB in California, but instead *Paracremonium* sp. I previously found in Vietnam (Fig. 3) (Lynch et al. 2016). We also are reporting a *Paracremonium* spp. recovered from KSHB infested plants in California that is closely related to *P. pembeum* which was not reported as a KSHB symbiont by Na et al. 2017. These fungi are not recovered frequently from female mycangia, but rather beetle larvae and gallery samples

(Freeman et al. 2015; Lynch et al. 2016). There also can be a significant difference in the proportion of these fungal propagules in isolation plating from different attacked hosts (Lynch et al. 2016). However, the function of this group is still unknown, but recovering these members from a native area such as Taiwan provides more evidence that the presence of these fungi may be important in ambrosia fungi symbiosis.

Ambrosia beetles from the *Euwallacea* genus have invaded the non-native areas on multiple occasions (CABI 2015; Eskalen et al. 2013; Hulcr and Dunn 2011, Hulcr and Stelinski 2017; Kasson et al. 2013; Mendel et al. 2012; O'Donnell et al. 2015; Paap et al. 2018; Stouthamer et al. 2017) and carry fungal symbionts that have evolved with them in mutualisms that provide the beetles with nutritional supplement from low-nutrient xylem tissue, while the fungi receive reliable dispersion and direct inoculation into plant hosts (Beaver, 1989). The present study suggests a non-exclusive relationship exists between *Euwallacea fornicatus* species complex members and closely related fungal species from specific clades within the genera: *Fusarium* spp. (AFC, Fig. 1), *Graphium* spp. (Fig.2, S1), and *Paracremonium* spp. (Fig. 3), which may all support nutritional requirements for the lifestyle of the insect vector and different stages of development (Freeman et al. 2013; Freeman et al. 2016). It is clear that AFC members, such as *F. euwallaceae* associated with PSHB, are the most prevalent in female mycangia (Kasson et al. 2013; Lynch et al. 2016), most likely to be recovered from gallery samples (Carrillo, Mayorquin, and Eskalen, *Unpublished data*), and lead to increased fecundity as a diet source over *G. euwallaceae* and *P. pembeum* in studies by Freeman et al. (2013; 2016). It was initially hypothesized from a diet experiment that *Euwallacea* spp., such as PSHB, require their specific *F. euwallaceae* associates to survive and reproduce (Freeman et al. 2013), but here we show evidence that the relationship may not be specific to a particular AFC species and possible that TSHB and PSHB are vectoring multiple AFC species present in Taiwan (Fig. 1). In a later diet experiment (Freeman et al. 2016), *G. euwallaceae* was found present significantly more in larvae and hypothesized to reduce competition between adults and developing larvae, but they could not survive or reproduce on *P. pembeum* alone. To investigate relationships of between *E. fornicatus* species complex members and their ambrosia fungi, future studies should test if they can survive on alternative fungi from AFC members, *Graphium* spp., and *Paracremonium* spp. in areas with multiple invasive *Euwallacea* spp. such as California and Florida in the United States.

The invasion of ambrosia beetles is a growing concern due to the ability of some invaders to attack and colonize healthy hosts with aid of their symbiotic partners. Of these invasive species *Xyleborus glabaratus*, causing

the disease Laurel wilt, is of concern because of the virulent nature of the plant pathogen *Raffalea lauricola* they vector which has led to the destruction of an estimated half billion native trees in affected regions (Hughes 2013). Similarly, *Euwallacea* spp. can attack and inoculate their plant pathogenic mutualists into healthy and declining trees (Hulcr and Stelenski 2017) and pose a threat to native, urban, and agricultural landscapes (Eskalen et al. 2013; O'Donnell et al. 2016). Plant pathogenic fungi can recombine to combat selective pressures such as host resistance genes and fungicide application, which has been reported in *Puccinia* spp. on wheat (Kim and Brewmaker 1977) as well as in *F. gramanarium* on wheat (Miedaner et al. 2001). When a compatible mating partner is introduced into areas with a clonal linage the result can yield higher virulence and resistance to host defenses and the rise of new clonal populations. We have shown here that native populations of *Euwallacea fornicatus* species complex members are vectoring different mating types of the heterothallic AFC species acquired from the region (Fig 1, 4; Table 3, 5). Of invasive AFC members, *F. euwallaceae* [AF-2] and *F. kuroshium* [AF-12], only one clonal population with mating type *MAT1-1-1* present in California where *Fusarium* spp. [AF-6] in Florida has both mating types present within the population based on the results of this study. The introduction of new mating types into areas into already affected areas may lead to increased diversity if these beetles and their mutualist fungal species encounter a new clonal lineage with a compatible mating type, which may be occurring in Florida since both *MAT1-1* and *MAT1-2* mating types are already present (Table 5). It should also be investigated if sexual recombination is indeed possible through sexual crosses within AFC species (and possibly *Graphium* spp. and *Paracremonium* spp) and the effects such events have on the vector. A diagnostic assay that can target the *MAT* genes of invasive AFC members provides a valuable tool for identifying the mating types of AFC species in existing and new invasions of *Euwallacea* spp.

As researchers explore deeper into ambrosia beetle symbiosis, the fungal-insect interactions continue to present more complexities as there are now potential fungal-fungal interactions with, at least, AFC Fusaria in these systems with potential for mating. How and if the ambrosia beetles have abilities to control this interaction or simply just survive with fungi and combinations they happen to encounter remains to be seen. It is apparent that other ambrosia beetle systems are promiscuous with their fungi (Carrillo et al. 2014; Hulcr and Cognato 2010; Kostovcik et al. 2015; Skelton et al. 2019), while some are apparently more specific (Batra 1985; Beaver 1989). The physical and/or molecular mechanisms which the ambrosia fungi are accumulated and purified in the mycangia should be further investigated to potentially find a weakness to exploit for control in invaded areas. The promiscuous nature of

members of the *E. fornicatus* species complex with their symbiotic ambrosia fungi continues to raise concern in invaded areas, such as California, and the potential for PSHB and KSHB complexes present in California to exchange plant pathogenic fungi and should alert local and government agencies for the effects this can have in an already destructive pathosystem.

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Figure Captions:

Fig. 1. Multilocus phylogenetic analysis of AFC fusaria conducted with four genes: ribosomal internal transcribed spacer (ITS), elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II largest subunit (RPB1), DNA-directed RNA polymerase II second largest subunit (RPB2). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from while the blue or red color pertains to the *MAT* type determined from each isolate

Fig. 2. Multilocus phylogenetic analysis of *Graphium* spp. conducted with two genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

Fig. 3. Multilocus phylogenetic analysis of *Paracremonium* spp. conducted with five genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II second largest subunit (RPB2), Calmodulin region (cmdA), and ATP citrate lyase region (alc1). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

Fig. 4. Agarose gel image of products from the AFC *MAT* type PCR assay. (A) shows products from heterothallic isolates containing the *MAT1-1-1* gene while (B) shows products from isolates containing *MAT1-2-1* gene. Lanes 2-13 represent AF-2, AF-12, AF-16, AF-13, AF-13, AF-15, AF-17, AF-14, AF-13, AF-18, AF-6, AF-8, respectively. Lane 14 is a non-treated control.

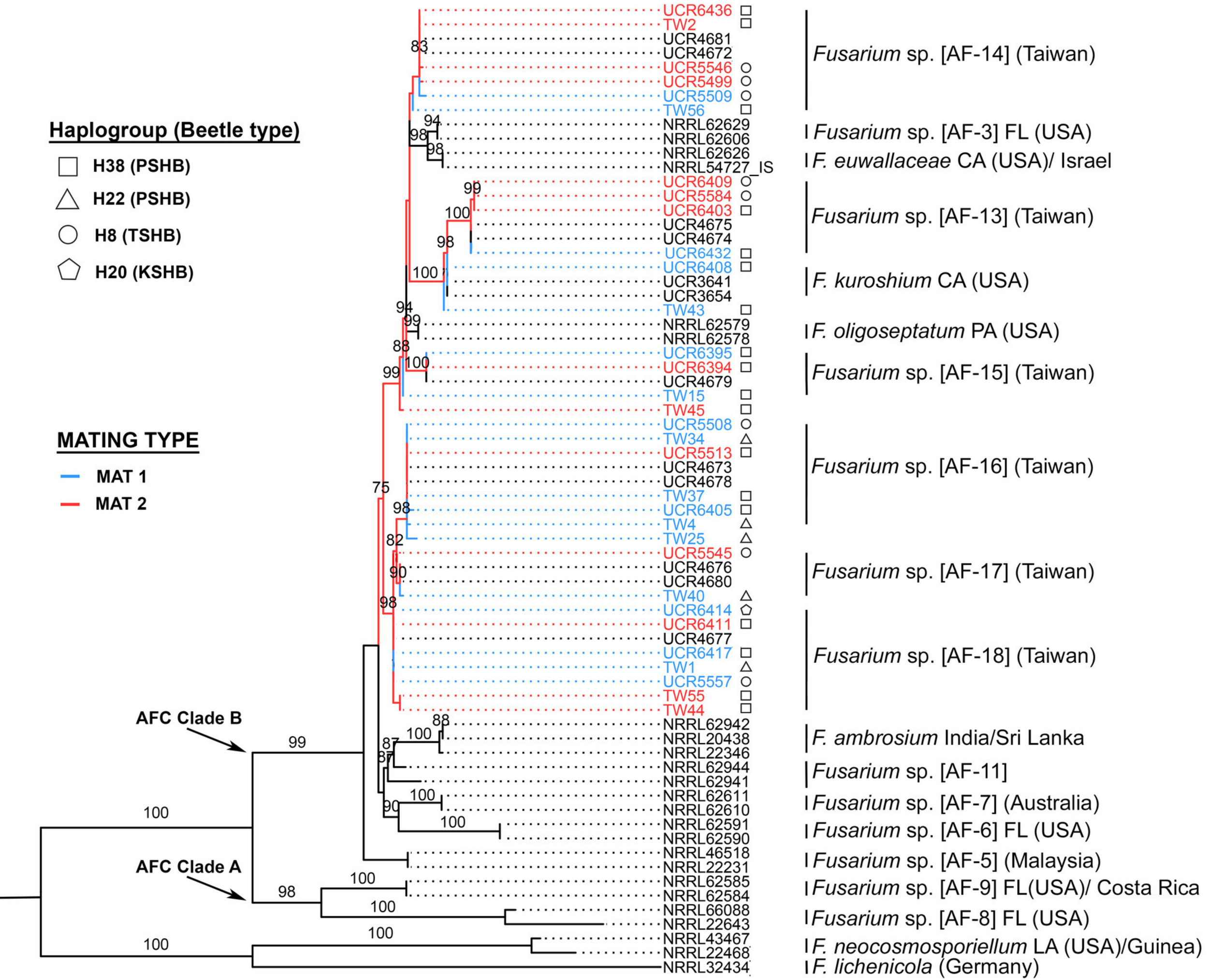
Supplemental Fig S1. Multilocus phylogenetic analysis of *Graphium* spp. focused on *Graphium* spp. associated with wood boring beetles conducted with two genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

Haplogroup (Beetle type)

- H38 (PSHB)
- H22 (PSHB)
- H8 (TSHB)
- H20 (KSHB)

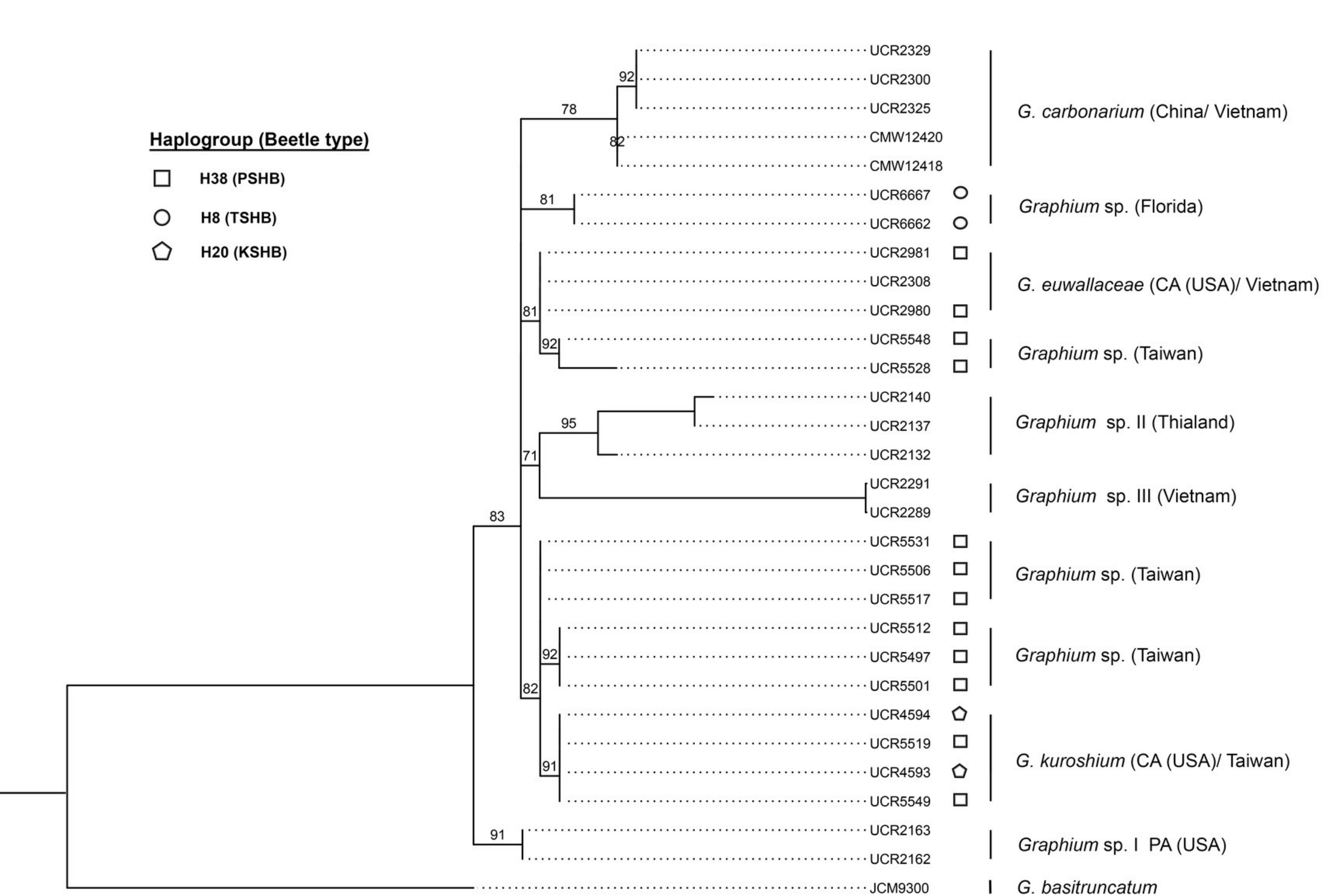
MATING TYPE

- MAT 1
- MAT 2



Haplogroup (Beetle type)

- H38 (PSHB)
- H8 (TSHB)
- H20 (KSHB)



Haplogroup (Beetle type)

- H38 (PSHB)
- H8 (TSHB)
- H20 (KSHB)

UCR6671
UCR6659
TW9
TW39
TW38
TW19
UCR2359
UCR2330
UCR2995
UCR2993
UCR7194
UCR7195
CBS485.77
CBS220.84
CBS188.82
CBS541.89
CBS137.35
Nectria_cinnabarina_CBS125165_T
Bifusarium_dimerum_CBS108944

90
97
91
100
93
100
100
87
100
95

Florida
Taiwan
Taiwan
Vietnam
CA (USA)
CA (USA)

***Paracremonium* sp. I**
P. pembeum
P. inflatum
Xenoacremonium recifei

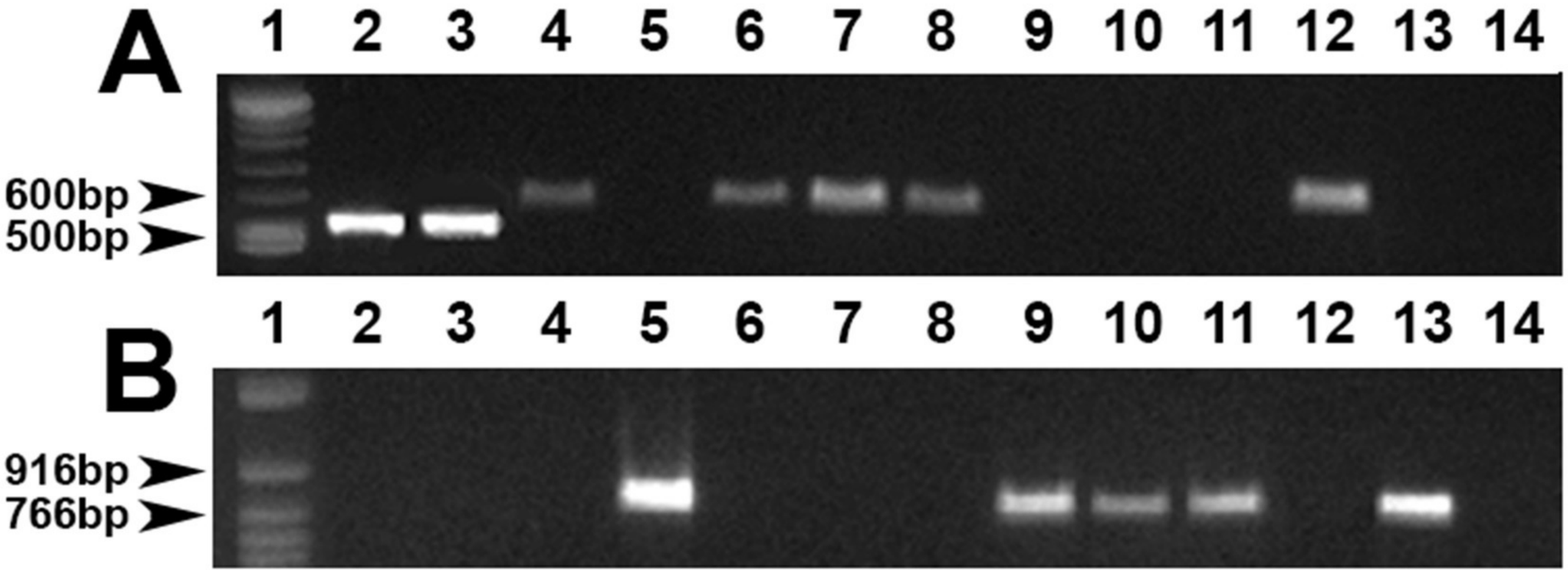

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
600bp
500bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
916bp
766bp

Table 1. Representative isolates of *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. from *Euwallacea* spp. and *Persea americana* obtained in this study

				GenBank accession numbers[ab]					
Isolate	**Species**	**Host**	**Location**	**ITS**	**EF1-α**	**RPB1**	**RPB2**	**cmdA**	**acl1**
TW1	*Fusarium* sp.	*Persea americana*	Danei District, Taiwan	MK432860	MK435437	MK435489	MK435521	…	…
TW2	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432862	MK435439	MK435491	MK435523	…	…
TW4	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432866	MK435443	MK435495	MK435527	…	…
TW15	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432861	MK435438	MK435490	MK435522	…	…
TW25	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432863	MK435440	MK435492	MK435524	…	…
TW34	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432864	MK435441	MK435493	MK435525	…	…
TW37	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432865	MK435442	MK435494	MK435526	…	…
TW40	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432867	MK435444	MK435496	MK435528	…	…
TW43	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432868	MK435445	MK435497	MK435529	…	…
TW44	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432869	MK435446	MK435498	MK435530	…	…
TW45	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432870	MK435447	MK435499	MK435531	…	…
TW55	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432871	MK435448	MK435500	MK435532	…	…
TW56	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432872	MK435449	MK435501	MK435533	…	…
UCR5499	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432873	MK435450	MK435502	MK435534	…	…
UCR5508	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432874	MK435451	MK435503	MK435535	…	…
UCR5509	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432875	MK435452	MK435504	MK435536	…	…
UCR5513	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432876	MK435453	MK435505	MK435537	…	…
UCR5545	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432877	MK435454	MK435506	MK435538	…	…
UCR5546	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432878	MK435455	MK435507	MK435539	…	…
UCR5557	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432879	MK435456	MK435508	MK435540	…	…
UCR5584	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432880	MK435457	MK435509	MK435541	…	…
UCR6394	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432881	MK435458	MK435510	MK435542	…	…
UCR6395	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432882	MK435459	MK435511	MK435543	…	…
UCR6403	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432883	MK435460	MK435512	MK435544	…	…
UCR6405	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432884	MK435461	MK435513	MK435545	…	…
UCR6408	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432885	MK435462	MK435514	MK435546	…	…
UCR6409	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432886	MK435463	MK435515	MK435547	…	…
UCR6411	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432887	MK435464	MK435516	MK435548	…	…
UCR6414	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432888	MK435465	MK435517	MK435549	…	…
UCR6417	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432889	MK435466	MK435518	MK435550	…	…
UCR6432	*F. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432890	MK435467	MK435519	MK435551	…	…
UCR6436	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432891	MK435468	MK435520	MK435552	…	…
UCR5497	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432903	MK435469	…	…	…	…
UCR5501	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432902	MK435470	…	…	…	…
UCR5506	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432901	MK435471	…	…	…	…
UCR5512	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432900	MK435472	…	…	…	…
UCR5517	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432899	MK435473	…	…	…	…
UCR5519	*G. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432898	MK435474	…	…	…	…
UCR5528	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432897	MK435475	…	…	…	…
UCR5531	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432896	MK435476	…	…	…	…
UCR5548	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432895	MK435477	…	…	…	…
UCR5549	*G. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432894	MK435478	…	…	…	…
UCR6662	*Graphium* sp.	*Euwallacea* sp.	FL (USA)	MK432893	MK435479	…	…	…	…
UCR6667	*Graphium* sp.	*Euwallacea* sp.	FL (USA)	MK432892	MK435480	…	…	…	…
TW9	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432907	MK435484	…	MK435556	MK435572	MK435564
TW19	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432904	MK435481	…	MK435553	MK435569	MK435561
TW38	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432905	MK435482	…	MK435554	MK435570	MK435562
TW39	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432906	MK435483	…	MK435555	MK435571	MK435563
UCR6659	*Paracremonium* sp.	*Euwallacea* sp.	FL (USA)	MK432908	MK435485	…	MK435557	MK435573	MK435565
UCR6671	*Paracremonium* sp.	*Euwallacea* sp.	FL (USA)	MK432909	MK435486	…	MK435558	MK435574	MK435566
UCR7194	*Paracremonium* sp.	*Euwallacea* sp.	CA (USA)	MK432910	MK435487	…	MK435559	MK435575	MK435567
UCR7195	*Paracremonium* sp.	*Euwallacea* sp.	CA (USA)	MK432911	MK435488	…	MK435560	MK435576	MK435568

[a]ITS = internal transcribed spacer region; EF1-α = translation elongation factor 1-α;

[b]RPB = DNA-directed RNA polymerase II subunit; cmdA = calmodulin; acl1 = ATP citrate lyase large subunit

Table 2. Descriptions and sequences of fungi obtained from GenBank used in the phylogenetic analyses

Isolate	Species	Host	Origin	GenBank accession numbers[ab]					
				ITS	EF1-α	RPB1	RPB2[c]	cmdA	acl1
NRRL20438	*Fusarium ambrosium*	*Euwallacea fornicatus*	India	AF178397	AF178332	JX171470	JX171584	...	...
NRRL22346	*F. ambrosium*	*E. fornicatus*	India	EU329669	FJ240350	KC691587	EU329503	...	...
NRRL62942	*F. ambrosium*	*Camellia sinensis*	Sri Lanka	KM406631	KM40662	KM406638	KM406645	...	...
NRRL54727	*F. euwallaceae*	*E. whifordiodendrus*	Israel	JQ038019	JQ038012	JQ038026	JQ038033	...	...
NRRL62626	*F. euwallaceae*	*Persea americana*	CA (USA)	KC691560	KU171722	KU171682	KU171702	...	...
UCR3641[c]	*F. kuroshium*	*Platanus racemosa*	CA (USA)	KX262196	KX262216	KX262236	KX262256	...	...
UCR3654	*F. kuroshium*	*P. americana*	CA (USA)	KX262201	KX262221	KX262241	KX262261	...	...
NRRL32434	*F. lichenicola*	*Homo sapien*	Germany	DQ094444	DQ246977	HM347156	EF470161	...	...
NRRL22468	*F. neocosmosporiellum*	*Arachis hypogaea*	Guinea	DQ094318	AF178349	KC691616	EU329512	...	...
NRRL43467	*F. neocosmosporiellum*	*H. sapien*	LA (USA)	EF453092	EF452940	HM347178	EF469979	...	...
NRRL62578	*F. oligoseptatum*	*E. validus*	PA (USA)	KC691565	KC691537	KC691595	KC691626,KC691655	...	...
NRRL62579	*F. oligoseptatum*	*E. validus*	PA (USA)	KC691566	KC691538	KC691596	KC691627,KC691656	...	...
UCR4674	*Fusarium* sp. [AF-13]	*Euwallacea sp.*	Taichung, Taiwan	KX262208	KX262228	KX262248	KX262268	...	...
UCR4675	*Fusarium* sp. [AF-13]	*Euwallacea sp.*	Taichung, Taiwan	KX262209	KX262229	KX262249	KX262269	...	...
UCR4672	*Fusarium* sp. [AF-14]	*Euwallacea sp.*	Taichung, Taiwan	KX262206	KX262226	KX262246	KX262266	...	...
UCR4681	*Fusarium* sp. [AF-14]	*Euwallacea sp.*	Taichung, Taiwan	KX262215	KX262235	KX262255	KX262275	...	...
UCR4679	*Fusarium* sp. [AF-15]	*Euwallacea sp.*	Taichung, Taiwan	KX262213	KX262233	KX262253	KX262273	...	...
UCR4673	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262207	KX262227	KX262247	KX262267	...	...
UCR4676	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262210	KX262230	KX262250	KX262270	...	...
UCR4677	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262211	KX262231	KX262251	KX262271	...	...
UCR4678	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262212	KX262232	KX262252	KX262272	...	...
UCR4680	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262214	KX262234	KX262254	KX262274	...	...
NRRL62590	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (USA)	KC691574	KC691546	KC691604	KC691635,KC691664	...	...
NRRL62591	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (USA)	KC691573	KC691545	KC691603	KC691634,KC691663	...	...
NRRL66088	*Fusarium* sp.	*Delonix regia*	FL (USA)	KM406632	KM40663	KM406639	KM406646	...	...
NRRL62941	*Fusarium* sp. [AF-10]	unknown	Singapore	KM406633	KM40662	KM406640	KM406647	...	...
NRRL62944	*Fusarium* sp. [AF-11]	*C. sinensis*	Sri Lanka	KM406634	KM40662	KM406641	KM406648	...	...
NRRL62606	*Fusarium* sp. [AF-3]	*Euwallacea* sp.	FL (USA)	KC691561	KC691533	KC691591	KC691622,KC691651	...	...
NRRL62629	*Fusarium* sp. [AF-3]	*E. interjectus*	FL (USA)	KC691564	KC691536	KC691594	KC691625,KC691654	...	...
NRRL22231	*Fusarium* sp. [AF-5]	*Hevea brasiliensis*	Malaysia	KC691570	KC691542	KC691600	KC691631,KC691660	...	...
NRRL46518	*Fusarium* sp. [AF-5]	*H. brasiliensis*	Malaysia	KC691571	KC691543	KC691601	KC691632,KC691661	...	...
NRRL62610	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691575	KC691547	KC691605	KC691636,KC691665	...	...
NRRL62611	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691576	KC691548	KC691606	KC691637,KC691666	...	...
NRRL62584	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (USA)	KC691582	KC691554	KC691612	KC691643,KC691672	...	...
NRRL62585	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (USA)	KC691577	KC691549	KC691607	KC691638,KC691667	...	...
NRRL22643	*Fusarium* sp. [AF-9]	*Xyleborus ferrigineus*	Costa Rica	KC691583	DQ247628	KC691613	KC691644,KC691673	...	...
JCM 9300	*Graphum basitruncatum*	Forest soil	Salomon Islands	AB038427	KJ131248	...	...	...	...
CMW12418	*G. carbonarium*	*Salix babylonica*	China	FJ434980	HM630602	...	...	...	...
CMW12420[c]	*G. carbonarium*	*S. babylonica*	China	FJ434989	HM630603	...	...	...	...
UCR2300	*G. carbonarium*	*A. auriculiformis*	Vietnam	KM592370	KM592362	...	...	...	...
UCR2325	*G. carbonarium*	*R. communis*	Vietnam	KM592372	KM592364	...	...	...	...
UCR2329	*G. carbonarium*	*R. communis*	Vietnam	KM592373	KM592365	...	...	...	...
UCR2308	*G. euwallaceae*	*Acacia auriculiformis*	Vietnam	KM592371	KM592363	...	...	...	...
UCR2980[c]	*G. euwallaceae*	*Persea americana*	CA (USA)	KF540224	KF534805	...	...	...	...
UCR2981	*G. euwallaceae*	*P. americana*	CA (USA)	KF540225	KF534806	...	...	...	...
CMW30626[c]	*G. fabiforme*	*Adansonia rubrostipa*	Madagascar	GQ200616	HM630592	...	...	...	...
CMW30627	*G. fabiforme*	*A. rubrostipa*	Madagascar	GQ200617	HM630593	...	...	...	...
CMW5605[c]	*G. fimbriisporum*	*Picea abies*	France	AY148177	HM630590	...	...	...	...
CMW5606	*G. fimbriisporum*	*P. abies*	Austria	AY148180	HM630591	...	...	...	...
UCR4593[c]	*G. kuroshium*	*P. americana*	CA (USA)	KX262276	KX262286	...	...	...	...
UCR4594[d]	*G. kuroshium*	*P. americana*	CA (USA)	KX262277	KX262287	...	...	...	...
CMW5601[c]	*G. laricis*	*Larix decidua*	Austria	AY148183	HM630588	...	...	...	...
CMW5603	*G. laricis*	*L. decidua*	Austria	AY148182	HM630589	...	...	...	...
CMW5292	*G. penicillioides*	*Populus nigra*	Czech Republic	HQ335310	HM630600	...	...	...	...
CMW5295	*G. penicillioides*	*P. nigra*	Czech Republic	HQ335311	HM630601	...	...	...	...
CMW12285	*G. pseudormiticum*	*Tsuga dumosa*	China	HM630608	HM630587	...	...	...	...
CMW503c	*G. pseudormiticum*	*Pinus* sp.	South Africa	AY148186	HM630580	...	...	...	...
UCR2159	*Graphium* sp. I	*Ailanthus altissima*	PA (USA)	KJ131228	KJ131238	...	...	...	...
UCR2162	*Graphium* sp. I	*A. altissima*	PA (USA)	KJ131231	KJ131241	...	...	...	...
UCR2163	*Graphium* sp. I	*A. altissima*	PA (USA)	KJ131232	KJ131242	...	...	...	...
UCR2132	*Graphium* sp. II	*Durio* sp.	Thailand	KM592367	KM363259	...	...	...	...
UCR2137	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131236	KJ131246	...	...	...	...
UCR2140	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131237	KJ131247	...	...	...	...
UCR2289	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592368	KM592360	...	...	...	...
UCR2291	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592369	KM592361	...	...	...	...
CBS485.77	*P. inflatum*	*H. sapien*	India	KM231829	KP012649	...	KM232395	KM231415	KM231065
UCR2993	*P. pembeum*	*Euwallacea* sp.	CA (USA)	KP012602	KP012642	...	KT936353	KT936374	KT936332
UCR2995	*P. pembeum*	*Euwallacea* sp.	CA (USA)	KP012604	KP012644	...	KT936355	KT936376	KT936334
UCR2330	*Paracremonium* sp. I	*R. communis*	Vietnam	KP030841	KP030849	...	KT936338	KT936359	KT936317
UCR2359	*Paracremonium* sp. I	*R. communis*	Vietnam	KP030842	KP030850	...	KT936339	KT936360	KT936318
CBS137.35	*X. recifei*	*H. sapien*	Brazil	KM231833	KM231968	...	KM232397	KM231419	KM231069
CBS188.82	*X. recifei*	*H. sapien*	Netherlands	KP012607	KP012647	...	KT936340	KT936361	KT936319
CBS541.89	*X. recifei*	Forest soil	Brazil	KM231834	KM231969	...	KM232398	KM231420	KM231070
CBS220.84	*Xenoacremonium recifei*	*P. americana*	CA (USA)	KP012608	KP012648	...	KT936341	KT936362	KT936320
CBS125165	*Nectria cinnabarina*	*Aesculus* spp.	France	MH856245	HM484656	...	KM232402	KM231424	KM231074
CBS108944	*Bifusarium dimerum*	*H. sapien*	Netherlands	JQ434586	KR673912	...	KM232363	KM231365	KM230996

[a]ITS = internal transcribed spacer region; EF1-α = translation elongation factor 1-α

[b]RPB = DNA-directed RNA polymerase II subunit; cmdA = calmodulin; acl1 = ATP citrate lyase large subunit

[c]Two accession numbers correspond to un-joined RPB2-1 and RPB2-2 sequences

[d]Denotes type-specimens

Table 3. List of the mating type (MAT) oligonucleotides developed targeting AFC species

Oligo ID	Sequence (5'→ 3')	T_m (°C)	GC (%)
AFC-MAT1F	GGTACATTGCCGAGATCAG	56.6	52.6
AFC-MAT1R	TATCTCCCTGAGTATGGACCT	55.3	47.6
AFC-MAT2F	CAACGACSTTGTTGCAG	54.3	52.9
AFC-MAT2R	CCAGGATCTGAGCTAAAGAA	54.7	45

Table 4. Representative beetle species proportion and AFC mating type proportion from collection sites in Taiwan

	***Euwallacea fornicatus* species complex**			**Mating type**[a]	
Field Collection Date	**PSHB (%)**	**TSHB (%)**	**KSHB (%)**	***MAT1-1-1* (%)**	***MAT1-2-1* (%)**
2017 log batch 1	92.6	4.9	2.5	…	…
2017 log batch 2	85.5	14.5	…	54.5	45.5
2017 log batch 3	78.2	18.7	3.1	60	40
2017 field batch	95	5	…	…	…
2018 field batch	97.5	2.5	…	33.3	66.6

[a] MAT assay was not performed on 2017 log batch 1 and 2017 field batch since fungi were not linked to beetle vector type

Table 5. Results from novel mating type assays targeting AFC Fusaria from Taiwan and other invaded areas

					Mating type		
Isolate	**Species**	**Host**	**Location**	**AFC Designation**	***MAT1-1-1***	***MAT1-2-1***	**GenBank Accession**
NRRL62942	*F. ambrosium*	*Camellia sinensis*	Sri Lanka	AF-1	+	-	MK463687
NRRL62626	*F. euwallaceae*	*Persea americana*	California, USA	AF-2	+	-	MK463688
UCR4511	*F. euwallaceae*	*Platanus racemosa*	California, USA	AF-2	+	-	MK463681
MB12	*F. oligoseptatum*	*E. validus*	Pennsylvania, USA	AF-4	+	-	MK463715
MB14	*F. oligoseptatum*	*E. validus*	Pennsylvania, USA	AF-4	+	-	MK463712
NRRL 22231	*Fusarium* sp.	*Hevea brasiliensis*	Malaysia	AF-5	+	-	MK463686
KOD133	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	+	-	MK463683
LL157	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	-	+	MK463716
UCR6638	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	+	-	MK463679
KOD1405	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-8	-	+	MK463713
UCR6665	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-8	-	+	MK463711
NRRL 66088	*Fusarium* sp.	*Delonix regia*	Florida, USA	AF-9	+	-	MK463685
NRRL 62941	*Fusarium* sp.	Unknown	Singapore	AF-10	+	-	MK463684
NRRL 62943	*Fusarium* sp.	*C. sinensis*	Sri Lanka	AF-11	-	+	MK463717
TW43	*F. kuroshium*	*P. americana*	Danei District, Taiwan	AF-12	+	-	MK463700
UCR6408	*F. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	AF-12	+	-	MK463692
UCR3641	*F. kuroshium*	*P. racemosa*	California, USA	AF-12	+	-	MK463689
UCR3645	*F. kuroshium*	*P. racemosa*	California, USA	AF-12	+	-	MK463680
UCR5584	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6394	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6403	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6409	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	MK463703
UCR6432	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	MK463705
TW2	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-14	-	+	…
TW56	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-14	+	-	MK463710
UCR5509	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	+	-	…
UCR5546	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	MK463702
UCR5499	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	…
UCR6436	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	MK463706
TW15	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-15	+	-	MK463695
TW45	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-15	-	+	MK463708
UCR6395	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-15	+	-	MK463690
TW1	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463696
TW34	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463697
TW37	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463698
UCR5508	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	+	-	…
UCR5513	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	-	+	…
UCR6405	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	+	-	MK463691
TW40	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-17	+	-	MK463699
UCR5545	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-17	-	+	…
UCR6414	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-17	+	-	MK463693
TW44	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-18	-	+	MK463693
TW55	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-18	-	+	MK463709
UCR5557	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	+	-	…
UCR6411	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	-	+	MK463704
UCR6417	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	+	-	MK463694
AFH1	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-hybrid species	+	-	MK463682
KOD1406	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-hybrid species	+	-	MK463714