Fungal Ecology 35 (2018) 42–50

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Methodological Advances

Specific and promiscuous ophiostomatalean fungi associated with Platypodinae ambrosia beetles in the southeastern United States

You Li [a], Yin-Tse Huang [a], Matthew T. Kasson [b], Angie M. Macias [b], James Skelton [a], Paige S. Carlson [c], Mingliang Yin [d], Jiri Hulcr [a, c, *]

[a] School of Forest Resources and Conservation, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA
[b] Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV, USA
[c] Department of Entomology and Nematology, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA
[d] College of Forestry and Landscape Architecture, South China Agricultural University, Guangdong, China

ARTICLE INFO

Article history:
Received 19 February 2018
Received in revised form
21 June 2018
Accepted 23 June 2018

Corresponding Editor: Nicolai Meyling

Keywords:
Ambrosia beetle
Ceratocystiopsis
Raffaelea
Forest pest
Symbiosis
Scolytinae

ABSTRACT

Ambrosia beetles in the subfamily Platypodinae (Coleoptera: Curculionidae) have been farming fungi for over 50 million y, yet they remain understudied and most of their fungal symbionts are unknown. We identified fungal communities associated with all four platypodine species native to the southeastern United States: *Euplatypus compositus*, *Euplatypus parallelus*, *Myoplatypus flavicornis*, and *Oxoplatypus quadridentatus*. Forty-eight samples were analyzed by quantitative culturing and DNA sequencing. Phylogenetic analyses of 28S rDNA sequences revealed that the four platypodines were routinely associated with several genera in the Ophiostomatales. *E. compositus* is associated primarily with *Raffaelea campbellii* 1 and *Raffaelea* sp. 6 and, to a lesser extent, *Raffaelea* sp. 2. *M. flavicornis* is associated with *Raffaelea* sp. 5. *E. parallelus* and *O. quadridentatus* are less specific; the latter mostly associated with *Raffaelea cyclorhipidia*. Three of the four beetle species were also associated with *Ceratocystiopsis* spp. This is the first report of *Raffaelea* associated with *E. parallelus*, which is invasive in Asia and Africa.

1. Introduction

The weevil subfamily Platypodinae comprises more than 1400 species, of which the vast majority are distributed in tropical regions and fewer than 10 species are found in wet temperate areas (Wood, 1993; Jordal, 2015). All but two species in the subfamily Platypodinae maintain nutritional symbioses with fungal cultivars (Jordal, 2014). Platypodinae and fungus-feeding Scolytinae have similar ecologies and together comprise the polyphyletic "ambrosia beetles". However, Platypodinae have a closer relationship with the weevil subfamily Dryophthorinae, which lacks fungal mutualists, than with the Scolytinae (McKenna et al., 2009; Gillett et al., 2014). The subfamily Platypodinae is estimated to have arisen in the mid-Cretaceous (119–88 Ma), much earlier than Scolytinae and the other fungus-farming insects (Jordal, 2015), and thus they represent the oldest known fungus-farming system.

As in scolytines, platypodine ambrosia beetles are typically attracted to dead or severely declining trees (Hubbard, 1896; Jordal, 2015). They cultivate communities of fungi in galleries in trees as the sole food for their larvae (Nobuchi, 1993; Jordal, 2014). Previous studies indicate that the symbiotic fungal communities from platypodines are dominated by ambrosia fungi in the genus *Raffaelea* (Ascomycota: Ophiostomatales) and other members of the Ophiostomatales, in addition to unrelated *Ambrosiozyma* yeasts (Beaver, 1989; Kubono, 2002; Belhoucine et al., 2011; Bellahirech et al., 2014; Dreaden et al., 2014; Musvuugwa et al., 2015; Yun et al., 2015; Hulcr and Stelinski, 2017). Symbiotic fungi function primarily as a nutritional resource but in several cases have been shown to be important plant pathogens (Six, 2003; Kobayashi and Ueda, 2005; Kinuura and Kobayashi, 2006). As vectors of pathogenic fungi, some platypodines are considered forest pests (Kile and Hall, 1988; Massoumi Alamouti et al., 2009; Inácio et al., 2012a). Their associated fungi cause staining of the wood around the galleries (Fig. 1), resulting in the downgrading of timber quality (Beaver, 2013). *Platypus quercivorus* and its fungal symbiont,

* Corresponding author. School of Forest Resources and Conservation, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA.
E-mail address: hulcr@ufl.edu (J. Hulcr).

https://doi.org/10.1016/j.funeco.2018.06.006
1754-5048/

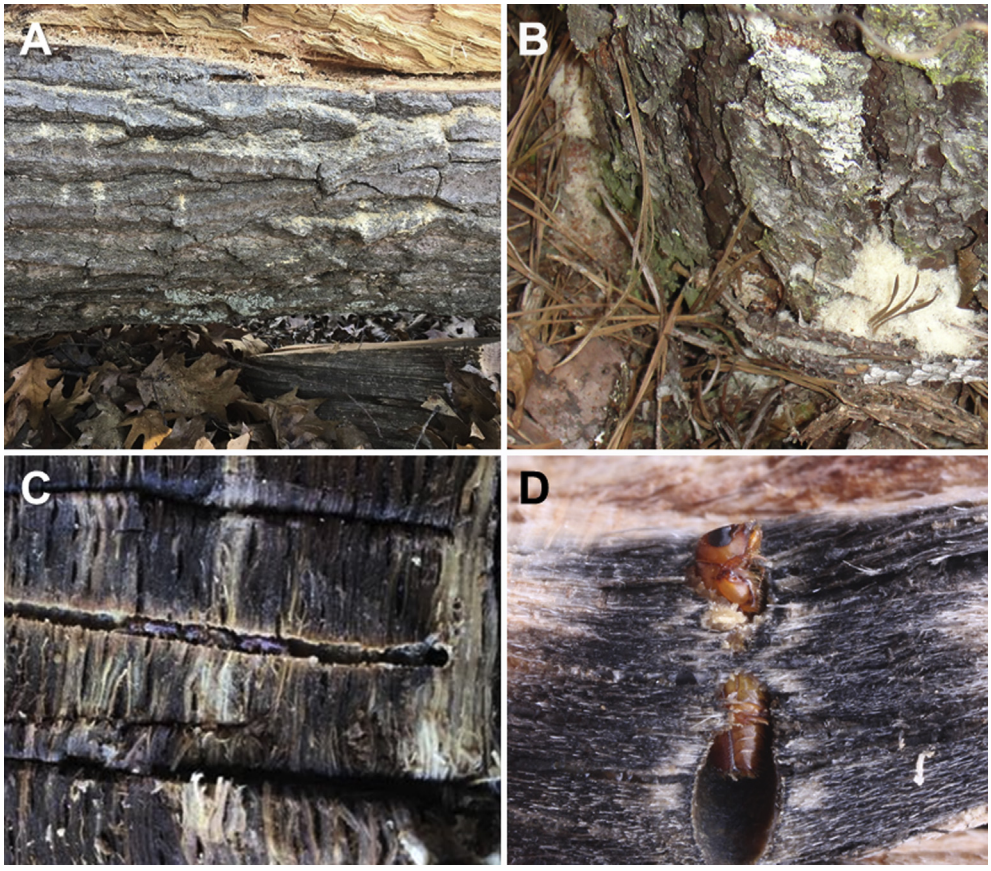

Fig. 1. Signs of platypodine infestation and associated ophiostomatalean fungal colonization. A. *Oxoplatypus quadridentatus* infesting a red oak (*Quercus rubra*); B. *Myoplatypus flavicornis* infesting a loblolly pine (*Pinus taeda*); C. associated fungal colonization and staining of surrounding *O. quadridentatus* galleries; and D. *Euplatypus parallelus* in gallery of *Acacia mangium* with associated staining of surrounding wood as a result of fungal colonization.

Raffaelea quercivora, cause significant mortality of oak trees in Japan (Kubono, 2002; Ito et al., 2003). Similarly, *Platypus cylindrus* is capable of killing European oaks, assisted by its nutritional symbiont *Raffaelea montetyi* (Belhoucine et al., 2011; Inácio et al., 2012a). *Megaplatypus mutatus*, which is associated with several *Raffaelea* species, attacks tree plantations in Argentina and Italy (Alfaro et al., 2007; Ceriani-Nakamurakare et al., 2016).

The study of ambrosia beetles and their symbiotic fungi is increasingly important as the international trade in lumber continues to grow and more exotic species are introduced (Brockerhoff et al., 2006). Although platypodines and their fungi have received some attention in Asia, Europe, and Oceania (Faulds, 1977; Kubono, 2002; Inácio et al., 2012a; Tarno et al., 2016), the fungal symbionts of the American fauna are poorly known (Batra, 1963; Farris and Funk, 1965; Ceriani-Nakamurakare et al., 2016). Compared with the high diversity of platypodine species in Asia, Africa, and the Neotropics, few species inhabit North America, with only seven species described to date (Wood, 1993). Four of these, *Euplatypus compositus*, *Euplatypus parallelus*, *Myoplatypus flavicornis*, and *Oxoplatypus quadridentatus*, are present in the southeastern United States (Atkinson, 2004).

The fungi associated with the four platypodines from the southeastern United States have not previously been studied. Two of these species, *M. flavicornis* and *O. quadridentatus,* are unusual in that their biology has never comprehensively been studied, let alone their relationships with symbiotic fungi. Based on previous collection information, *M. flavicornis* usually infests recently dead pine trees (*Pinus*) along with pine-feeding bark beetles (Curculionidae: Scolytinae), whereas *O. quadridentatus* prefers oak (Fagaceae) (Atkinson and Peck, 1994; Atkinson, 2004). *E. compositus* and *E. parallelus* are locally common and easily attracted to light traps. In the few reports of fungal isolations from southeastern platypodines, Batra (1963) and Verrall (1943) isolated the yeast fungus *Ambrosiozyma monospora* from *E. compositus* in Mississippi. *E. parallelus* is native throughout the tropical and subtropical regions in the Americas, but it has recently become an invasive species internationally and is now found throughout Africa, Asia, and parts of Oceania (Beaver, 2013; Gillett and Rubinoff, 2017). This beetle species has been reported to attack living rubber trees *Hevea brasiliensis* in Brazil (Pereira da Silva and Putz, 2013) and China (Li et al., 2018), as well as Indian rosewood, *Dalbergia sissoo,* in Bangladesh (Boa and Kirkendall, 2004). Several reports from Asia indicated it as a suspected vector of fungal pathogens of Burmese rosewood *Pterocarpus indicus* (Sanderson et al., 1996; Boa and Kirkendall, 2004; Bumrungsri et al., 2008; Tarno et al., 2016).

Studying the fungal symbionts of platypodine species is difficult for three reasons: (1) Platypodine species often colonize deep within the lower trunk of large trees (Fig. 1A) (Atkinson and Peck, 1994), making the collection of these beetles laborious. (2) The presence and location of mycangia (organs where the nutritional symbiont is concentrated and transported to new host trees) of most platypodine species are uncertain despite the presence of superficial pronotal pits on several of these species (Nakashima, 1975; Wood, 1993; Hulcr and Stelinski, 2017). (3) The phylogenetic placement of their most commonly reported fungal symbionts in the Ophiostomatales has not fully been resolved, leading to

Table 1
Collection of platypodine beetles.

Species	Location	Source	Date	Number of specimens
Euplatypus compositus	Gainesville, FL, USA	Light trap	2015-III-15	4
	Gainesville, FL, USA	Oak	2015-VIII-27	4
	Gainesville, FL, USA	Light trap	2017-III-15	2
	Cleveland, GA, USA	Light trap	2017-VII-18	1
Euplatypus parallelus	Miami, FL, USA	Light trap	2015-VIII-14	5
Myoplatypus flavicornis	Gainesville, FL, USA	Pine	2015-VII-9	5
	Gainesville, FL, USA	Pine	2016-V-4	3
	Gainesville, FL, USA	Pine	2017-III-5	5
Oxoplatypus quadridentatus	Gainesville, FL, USA	Oak	2015-VII-28	5
	Front Royal, VA, USA	Oak	2016-XI-24	9
	Washington, D.C., USA	Oak	2016-IX-26	5

taxonomic uncertainty of recovered fungi. In contrast, scolytine beetles and their relationships with fungi are better known (Hulcr and Stelinski, 2017; Vanderpool et al., 2017).

For the first time, we identified the fungal symbionts of all four Platypodinae species in the southeastern United States by using culturing techniques and community analyses. We explored the phylogenetic relationships among recovered ophiostomatalean fungi to accurately place our isolates among previously sequenced and analyzed isolates from platypodines as well as among ophiostomatalean fungi previously recovered from a diverse group of Scolytinae (Bumrungsri et al., 2008; Inácio et al., 2012b; Tarno et al., 2016).

2. Materials and methods

2.1. Beetle collection

Forty-eight samples representing four species of platypodine beetles native to the southeastern United States, *E. compositus, E. parallelus, M. flavicornis,* and *O. quadridentatus*, were collected from 2015 to 2017 (Table 1). To decrease the effect of opportunistic and transient commensal fungi that may have cross-contaminated samples through shared trees, every beetle sample was acquired from at least two locations or more than two trees from the same area, except for *E. parallelus*. Specimens that came from the same field sites were taken from different plant individuals at least 100 m apart. When possible, both adult males and females were taken from galleries and grouped in one sample. The beetle specimens were stored alive at room temperature with water-moistened sterile paper towels for 1 d after collection.

2.2. Fungal isolation

Isolation of fungi from ambrosia beetles is usually most efficient from their mycangia (Bateman et al., 2016), but some Platypodinae lack superficial exoskeletal pits on the pronotum and it is unknown whether there are any internal mycangia (Fig. 2A). Therefore, sampling focused on recovering fungi separately from beetle heads, pronota, and surface washes. For galleries, only active galleries with developing larvae were sampled.

To decrease the amount of non-specific fungi superficially attached to the exoskeleton of trap-caught beetles, live beetles were first washed by vortexing for 10 s in sterile water. A second wash was performed using a solution of 1 ml water and one drop of Tween 80. Beetles were held with forceps under a dissecting microscope while the head, thorax, and abdomen were separated using a sterile scalpel as previously described (Fraedrich et al., 2008; Kasson et al., 2013). The head and pronotum were transferred into 2 ml microcentrifuge tubes containing 1 ml phosphate buffer solution and crushed using sterilized micropestles. The tube containing a macerated body segment and second wash was then serially diluted and plated at concentrations of 1/10, 1/100, and 1/1000 on potato dextrose agar medium as described by Li et al. (2015). The medium was amended with 0.05 g/L cycloheximide to limit growth of non-ophiostomatalean fungi (Harrington, 1981). Colony forming units (CFUs) were counted for each morphotype. If the beetle was collected from wood, the surface layer of the gallery was scraped and plated. Fungi were allowed to grow at 25 °C for

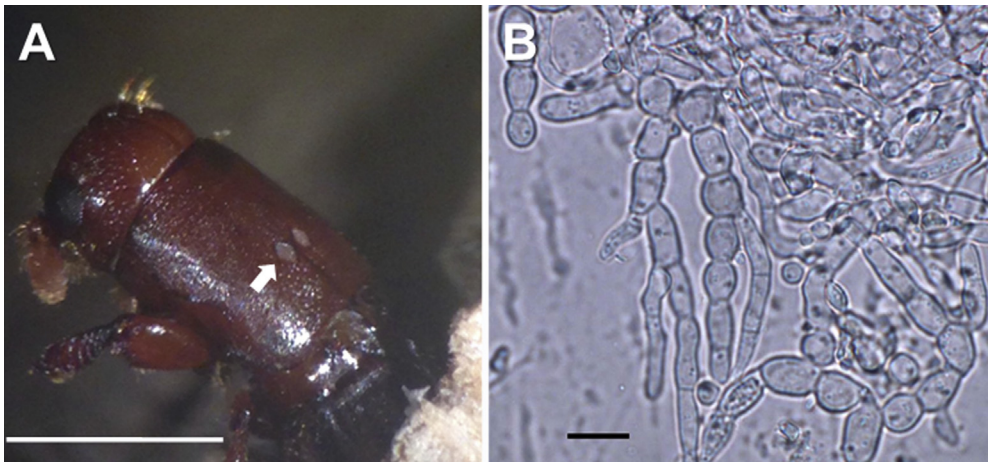

Fig. 2. A. Paired pronotal pits (white arrow) of adult female of *O. quadridentatus* and B. associated budding fungal hyphae recovered from a single pit. Scale bars are as follows: A, 1 mm; B, 10 μm.

5–7 d. Representative isolates of predominant fungal morphotypes recovered across the four platypodine beetle species were retained for molecular identification.

2.3. DNA extraction, amplification, and sequencing

Extraction of genomic DNA from fungal cultures was performed by scraping 5–10 μg tissue from pure cultures and adding it to 20 μl extraction solution from the Extract-N-Amplify Plant PCR kit (Sigma-Aldrich). Samples were then incubated at 96 °C for 30 min. Following incubation, 20 μl of 3% bovine serum albumin solution was added, and the mix was vortexed and spun down. The upper 20 μl of the supernatant was used as the PCR template.

PCR amplification was performed on portions of the 28S large subunit (LSU) ribosomal DNA (rDNA) loci using the primer pair LR0R/LR5 (Vilgalys and Hester, 1990). Final PCR volumes of 25 μl consisted of 1 μl template DNA, 1 μl each of forward and reverse primer, 1 μl dimethyl sulfoxide, 12.5 μl Premix Taq™ (Ex Taq™ Version 2.0; Takara Bio, Inc.), and 9.5 μl sterile water. Excess primers and dNTPs in the amplified products were removed using the ExoSAP-IT kit (Affymetrix, Inc.) according to the manufacturer's instructions. Sanger sequencing was performed by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL) or GENEWIZ (South Plainfield, NJ).

2.4. Fungal identification, taxa assignment, and phylogenetic analyses

Sequence chromatograms were inspected for quality and assembled in Geneious 9.1.5 (https://www.geneious.com/). Identification of sequences was first made to the lowest reliable taxonomic rank via NCBI BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of sufficient length and quality were selected and binned into operational taxonomic units (OTUs) at 100% similarity. The fungi identified as ophiostomatalean were further classified using phylogenetic analysis because these fungi are known to be widely crucial to platypodine beetle biology and because this group is not well represented or curated in GenBank. We also reconfirmed that the morphology of each isolate corresponded to its molecular identity. For isolates that were lost to contamination, which is not uncommon among slow-growing *Raffaelea* and close allies, identification was inferred by sequences of representatives from the same morphotype (e.g., two isolates from *E. parallelus*).

Reference LSU sequences from representative *Raffaelea* species (Simmons et al., 2016) were included in phylogenetic analyses (Supplementary Table 1). The Akaike information criterion in jModeltest 2.1.10 (Guindon and Gascuel, 2003; Posada, 2008) was used to select the nucleotide substitution model GTR + I + G. Bayesian inference analyses were performed using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003), and maximum likelihood analyses were performed using RAxML2.0 (Stamatakis, 2014) on the University of Florida supercomputer (HiperGator2.0). The tree was edited in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) and Adobe® Photoshop® CS4. Isolate names were created based on monophyly and closest relative. Representative sequences for all new taxa were uploaded to GenBank (accession no. LC363534–LC363555).

2.5. Data analysis

Fungal CFU and frequency data were processed using Microsoft Office Excel 365 ProPlus. For specimens collected from wood, we counted all the fungal isolations of each male and female pair together as one sample in CFU and frequency statistics. In our mycobiota, as well as quantitative analysis, different species of Saccharomycetales yeasts (*Candida* spp., *Pichia* spp., or *Ambrosiozyma* spp.) and Hypocreales (*Fusarium* spp.) were lumped in each category without further identification to genus or species level. Although ubiquitous in beetle fungal communities, yeast and *Fusarium* spp. are not primary mutualists (with the exception of the ambrosial *Fusarium* associated with *Euwallacea*, unrelated to platypodine beetles; Kasson et al., 2013; Kostovcik et al., 2015; Musvuugwa et al., 2015; Yun et al., 2015; Bateman et al., 2016; Ceriani-Nakamurakare et al., 2016; Hulcr and Stelinski, 2017). The yeast genus *Ambrosiozyma* contains putative associates of platypodine beetles, but our morphotype screening approach did not have the necessary resolution to reliably distinguish *Ambrosiozyma* species from other yeasts. Fungal morphotypes found in only a single beetle were not included.

3. Results

3.1. Sequencing and phylogenetic analysis

In total, 66 ophiostomatalean fungal isolates were recovered from 48 platypodine beetles across the southeastern United States. Based on 100% sequence similarity with known LSU sequences, 44 fungal isolates representing 12 fungal OTUs in *Raffaelea* were recovered, along with two fungal isolates representing two fungal OTUs in *Esteya*, one fungal isolate representing one fungal OTU in *Leptographium*, eight fungal isolates representing two OTUs in *Ophiostoma*, and 11 isolates representing four OTUs in *Ceratocystiopsis* (Fig. 3; Table 2).

Nineteen ophiostomatalean species spanning five genera were identified (Table 2). All *Raffaelea* isolates belonged to the *Raffaelea s. str.* clade except for one isolate of *Raffaelea* sp. 8, which belonged in the *Raffaelea sulphurea* complex. Every platypodine species was associated with at least one *Raffaelea* species. None of the fungi recovered belonged to the *Raffaelea lauricola* complex, members of which are known to cause the devastating laurel wilt (de Beer and Wingfield, 2013).

3.2. Fungal community analysis

E. compositus and *O. quadridentatus* were associated with the greatest diversity of *Raffaelea* species, each with four *Raffaelea* species. In *E. compositus*, two species, *Raffaelea* cf. *campbellii* 1 and *Raffaelea* sp. 6 appeared to be highly specific, as both were present in beetles from Georgia and Florida, and both were obtained in Florida during every collection. *R.* cf. *campbellii* 1 appeared conspecific with *R. campbellii s. str.* in our LSU-based phylogeny, but a previous analysis of this isolate by Simmons et al. (2016) using additional markers determined that our isolate is divergent from *R. campbellii s. str.*

The fungi of *O. quadridentatus* were less specific: all *Raffaelea* spp. were isolated in low quantities and CFUs. Only *Raffaelea cyclorhipidia* appeared in collections from three locations (D.C., Florida, and Virginia) but in low frequency (Tables 2 and 3). *Ceratocystiopsis* sp. 1 and *Raffaelea* cf. *campbellii* 2 had a relatively high frequency in the D.C. and Virginia collections, respectively (Table 2).

E. parallelus was associated with only one *Raffaelea, Raffaelea* sp. 7, which was isolated from all individuals (Tables 2 and 3), though our sample was limited to five beetles from one location in south Florida. *M. flavicornis* was associated with three *Raffaelea* species, among which *Raffaelea* sp. 5 appeared with a relatively high frequency (Table 3) and was obtained every year in Florida since 2014.

Esteya and *Leptographium* sporadically appeared in *O. quadridentatus* and *M. flavicornis*. Two *Ophiostoma* spp. were associated with *O. quadridentatus* and *M. flavicornis* but appeared at only one location or in only one year. Two unknown species of

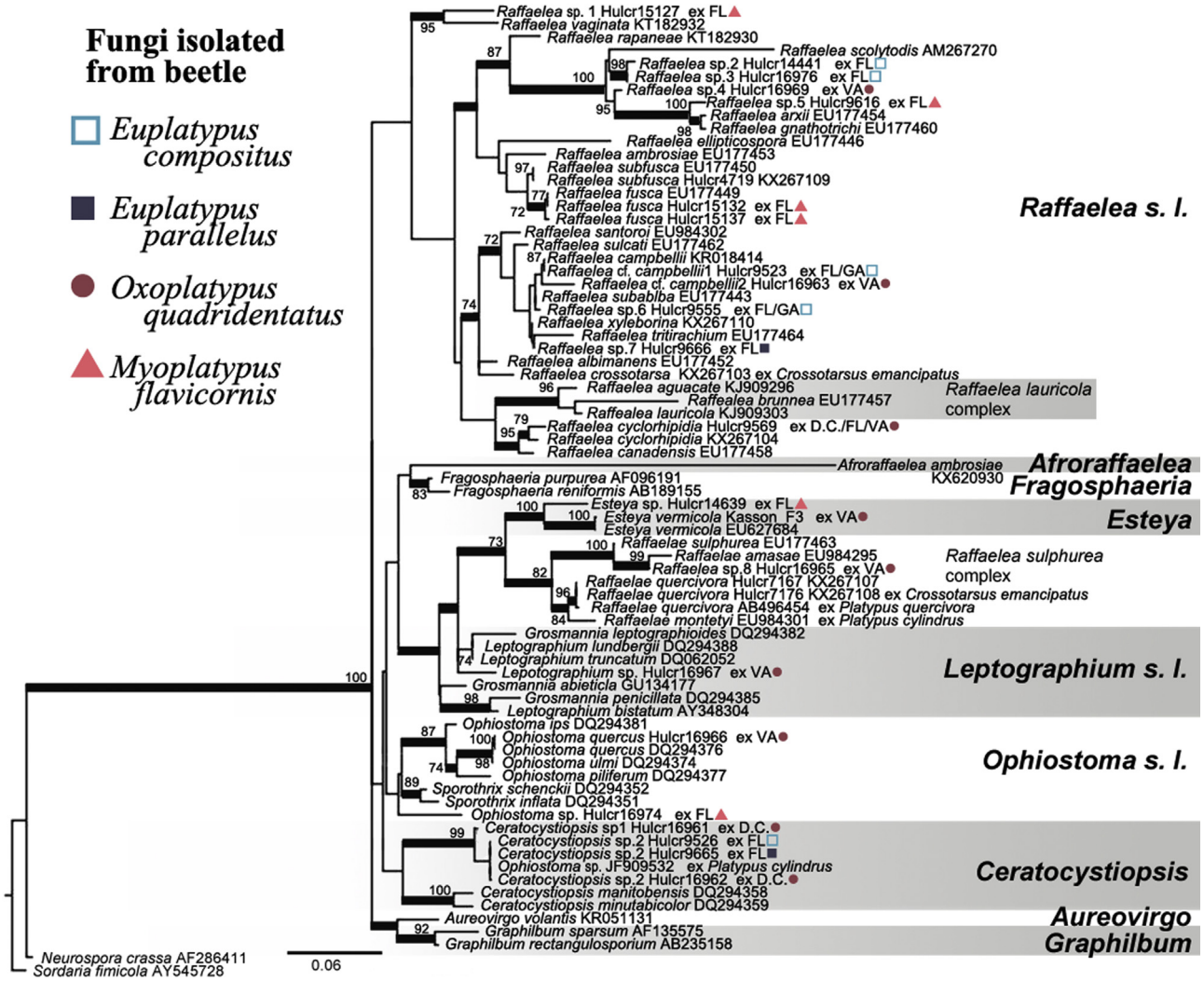

Fig. 3. Best maximum likelihood tree from RAxML analysis of 28S rDNA. Values at nodes represent maximum likelihood bootstrap percentages ≥70% from a summary of 500 replicates, and branches in bold represent bootstrap percentages ≥95%.

Ceratocystiopsis were recovered from all platypodine species except *M. flavicornis*. *Ceratocystiopsis* sp. 2 was recovered from three beetle species, whereas *Ceratocystiopsis* sp. 1 was only associated with *O. quadridentatus* in Washington, D.C.

Fusarium was recovered from two platypodine species but was less common than *Raffaelea*. Saccharomycetales yeasts (*Candida* spp., *Pichia* spp., and *Ambrosiozyma* spp.) were abundant and present in most individuals.

4. Discussion

We report preliminary evidence of consistent and widespread species-level associations between platypodines and ophiostomatalean fungi, particularly *Raffaelea*. The oldest record of platypodine–ophiostomatalean association in North America appears to be a fungus resembling *Tuberculariella* sp. (transferred to *Raffaelea* by Harrington et al., 2010) that was isolated from the beetle *Treptoplatypus wilsoni* (Farris and Funk, 1965). Subsequent studies also routinely reported *Raffaelea* spp., but none was systematic enough to allow for quantification of the strength of the association (Payton, 1989; Kinuura, 2002; Bellahirech et al., 2014; Dreaden et al., 2014). In some cases, *Raffaelea* isolated here were also previously reported from single platypodine species at different locations and times, such as two species of *Raffaelea* (sp. 6 and cf. *campbellii* 1) recovered from *E. compositus* in Florida and Georgia, and *Raffaelea* sp. 5 from *M. flavicornis* for 3 y. These repeated reports, corroborated here, suggested that *R.* cf. *campbellii* 1 and *Raffaelea* sp. 6 are the primary fungi of *E. compositus* and *Raffaelea* sp. 5 is the primary fungus of *M. flavicornis*. These three putative species were also isolated from their vectors with high frequency and yielded abundant CFUs from each individual.

E. parallelus is of particular importance because of its rapid global spread and its ability to vector wilt pathogens in Asia and Oceania (Bumrungsri et al., 2008; Beaver, 2013; Gümüş and Ergün, 2015; Gillett and Rubinoff, 2017). Neither *Raffaelea* nor any other ophiostomatalean species were found during previous isolations of *E. parallelus* (Bumrungsri et al., 2008; Tarno et al., 2016) and *Euplatypus segnis* (Alvidrez-Villarreal et al., 2012). This is the first study to use both quantitative culturing and Ophiostomatales-selective media, and we found that *Raffaelea* sp. 7 is associated with *E. parallelus* in Florida. Although our collections are limited to a single location in the United States, *Raffaelea* sp. 7 also appeared in *E. parallelus* from Hainan, China (Li et al., unpublished data). The appearance of the same fungus in two geographically separate collections further suggests that the relationship between *Raffaelea* sp. 7 and *E. parallelus* is strong. The previous failure to isolate

Table 2
Cumulative frequency of fungal isolation from four platypodine beetle species.[a]

	Euplatypus compositus		*Euplatypus parallelus*	*Oxoplatypus quadridentatus*			*Myoplatypus flavicornis*
	GA, USA (1)	FL, USA (10)	FL, USA (5)	D.C., USA (5)	FL, USA (5)	VA, USA (9)	FL, USA (13)
Ophiostomatales							
Raffaelea							
Raffaelea cf. *campbellii* 1	1	4					
Raffaelea cf. *campbellii* 2						5	
Raffaelea cyclorhipidia				2	1	2	
Raffaelea fusca							2
Raffaelea sp. 1							3
Raffaelea sp. 2		3					
Raffaelea sp. 3		1					
Raffaelea sp. 4						1	
Raffaelea sp. 5							8
Raffaelea sp. 6	1	4					
Raffaelea sp. 7			5				
Raffaelea sp. 8						1	
Esteya							
Esteya vermicola						1	
Esteya sp.							1
Leptographium							
Leptographium sp.						**1**	
Ophiostoma							
Ophiostoma cf. *quercus*						**3**	
Ophiostoma sp.							**5**
Ceratocystiopsis							
Ceratocystiopsis sp. 1				**5**			
Ceratocystiopsis sp. 2		**3**	2	1			
Hypocreales							
Fusarium spp.			**2**				**7**
Saccharomycetales							
Candida spp./*Pichia* spp./*Ambrosiozyma* spp.	1	6	5	3	5	5	8

[a] Note: Numbers in bold indicate this fungus appeared only from the beetle's surface and gallery; numbers and locations with underline indicate this fungus appeared in various time from the same location (single underline = two times; double underline = three times); numbers in parentheses after states mean total individuals we isolated from each location.

Table 3
Colony forming unit range and frequency of fungal isolation.[a]

	***Euplatypus compositus* (N = 11)**	***Euplatypus parallelus* (N = 5)**	***Oxoplatypus quadridentatus* (N = 19)**	***Myoplatypus flavicornis* (N = 13)**
Ophiostomatales				
Raffaelea cf. *campbellii*	20–4,000			
Raffaelea sp. 6	80–3,000			
Raffaelea sp. 2	100			
Ceratocystiopsis sp. 2	100	100–1,000	20	
Raffaelea sp. 3	100			
Raffaelea sp. 7		200–3,000		
Ceratocystiopsis sp. 1			90–1,500	
Raffaelea cf. *campbellii*			19–190	
Raffaelea cyclorhipidia			20–380	
Ophiostoma cf. *quercus*			100	
Raffaelea sp. 4			80	
Raffaelea sp. 8			N/A	
Esteya vermicola			10–40	
Leptographium sp.			N/A	
Raffaelea sp. 5				130
Ophiostoma sp.				N/A
Raffaelea sp. 1				10–30
Raffaelea fusca				30–100
Esteya sp.				200
Hypocreales				
Fusarium spp.		N/A		N/A
Saccharomycetales				
Candida spp./*Pichia* spp./*Ambrosiozyma* spp.	40–7,000	100-3,000	5–4,700	40–1,400

Frequency (%)
0–10
11–20
21–30
31–40
41–50
51–60
61–70
91–100

[a] Note: Cell color indicates the frequency. N/A = not applicable.

Raffaelea from *E. parallelus* is likely attributable to the use of non-selective, nutrient-rich media, where *Fusarium* and yeasts rapidly colonize the media, outcompeting the ophiostomatalean fungi. When isolations were performed on non-selective media, we also isolated abundant *Fusarium* and yeasts from *E. parallelus* (Li et al., unpublished data). Thus we conclude that *Raffaelea* sp. 7 is the likely primary nutritional mutualist of *E. parallelus*.

One *Raffaelea* species, *R. cyclorhipidia*, was consistently present with *O. quadridentatus* at different locations. Compared with *R.* cf. *campbellii* 2, *R. cyclorhipidia* is the more likely mutualist of *O. quadridentatus,* though this fungus was rarely isolated by us. *O. quadridentatus* specializes on diseased but still living oaks, similar to several other platypodine pests including *P. quercivorus*, *P. cylindrus,* and *Platypus koryoensis,* whose symbiotic fungi belong to the *R. sulphurea* complex (Kubono, 2002; Kim et al., 2009; Belhoucine et al., 2011; Simmons et al., 2016). Our isolations indicate that the primary fungal associate of *O. quadridentatus* is not in that complex, even if the woody host range of the beetle is similar.

The phenomenon of multiple *Raffaelea* species being associated with a single beetle species has previously been recorded in the Platypodinae, such as in *M. mutatus*, *P. quercivorus*, *P. cylindrus*, *P. koryoensis*, *Platypus hamatus,* and *Dinoplatypus flectus* (Endoh et al., 2011; Inácio et al., 2012a; Ceriani-Nakamurakare et al., 2016; Skelton et al., 2018; Park, pers. comm.). However, this pattern should not be equated with lack of specificity. Instead, it appears that some non-specific *Raffaelea* species are widespread across several unrelated scolytine beetle tribes including Corthylini and Xyleborini, while other *Raffaelea* species are more specific and dominate their respective beetle vectors (Harrington et al., 2011; Biedermann et al., 2013; Kasson et al., 2013; Bateman et al., 2015; Simmons et al., 2016; Ploetz et al., 2017). The lack of species-level fidelity among ambrosia beetles in general has been attributed to small mycangia (Nakashima, 1975; Nobuchi, 1993). Many platypodines have small and variable mycangia including exoskeletal pores and internal sacs (Nakashima, 1975, 1979, 1982; Nobuchi, 1993). On the other hand, ambrosia beetle groups with large mycangia have been suggested to be highly specific to a single species of their fungal symbiont (Mayers et al., 2015). Although the morphology and location of mycangia may play a role in the fidelity of the symbiosis, we also note that most ambrosia beetle groups vectoring *Raffaelea* display a lack of fidelity, whereas the symbiont-specific beetles vector fungi belonging to other groups (Harrington et al., 2011; Kostovcik et al., 2015). Therefore, specificity may be a feature of the fungus ecology rather than of the beetle morphology.

The analysis relies on the use of a single marker. The LSU region may not distinguish all species of Ophiostomatales, however in this dataset, its three variable regions were informative enough to distinguish different fungal communities between beetle species and classify most isolates to the species level. In addition, the LSU phylogeny presented here is almost completely congruent with the most recent LSU- and ITS- based phylogenies of Ophiostomatales (de Beer and Wingfield, 2013; Brown et al., 2014).

Ophiostomatalean fungi associated with bark beetles can be variable in their spatial occurrence and seasonal succession (Linnakoski et al., 2016). However, most *Raffaelea* species are obligate associates of their beetle vectors, and typically occur only in the wood that is well-colonized by the beetles (Hulcr and Stelinski, 2017). While the season may have an influence on the fungus abundance in the wood, the beetle-fungus specificity does not seem to deviate with seasons.

In some platypodines, the location of the mycangia is yet to be discovered. In this study, only *E. compositus* and *O. quadridentatus* have visible exoskeletal pores on the pronotum (Fig. 2), whereas *E. parallelus* and *M. flavicornis* lack the pores. It is possible that the latter two species have an unknown internal mycangium; our culturing did not reveal any particular body part with a conspicuous concentration of CFUs, hinting at a possible occurrence of an internal mycangium. Non-destructive 3-D visualization of mycangia is now possible, and recent studies have helped distinguish the pharyngeal mycangia of *Premnobius cavipennis* (Scolytinae: Ipini) from the pre-oral mycangia found in the distantly related Xyleborini ambrosia beetles (Bateman et al., 2017). This new technique may prove useful for identifying and describing mycangia in platypodines.

We demonstrated that platypodine beetles, *E. compositus*, *E. parallelus,* and *O. quadridentatus*, have a moderate association with *Ceratocystiopsis* spp. *Ceratocystiopsis* is a genus of morphologically and molecularly distinct Ophiostomatales that are strongly associated with bark beetles and separate from the genus *Ophiostoma* (Zipfel et al., 2006; Seifert et al., 2013). This genus appeared in three of the four studied beetle species. Previous reports have confirmed the presence of *Ceratocystiopsis* with platypodine beetles. Inácio et al. (2012b) and Bellahirech et al. (2014) recovered *Ophiostoma* sp. (GenBank accession no. JF909532) from *P. cylindrus* that aligned with *Ceratocystiopsis* sp. 2 in our phylogenetic analysis (Fig. 3). The specificity and ecological significance of the association between *Ceratocystiopsis* and platypodine beetles await further investigation.

The Saccharomycetales yeasts accounted for a significant proportion of the fungal community in the four studied platypodine beetle species, which agrees with other recent studies of platypodine beetles (Yun et al., 2015; Tarno et al., 2016; Skelton et al., 2018). Preliminary results of co-culturing *Raffaelea* and some yeasts indicate that they are able to coexist (Yun et al., 2015). This compatibility would allow both to grow together in the galleries of bark beetles (Davis, 2015). *Ambrosiozyma* is a unique genus in Saccharomycetales yeasts because most of the species have been isolated exclusively from ambrosia beetles, hence the origin of the genus name (Kurtzman and Robnett, 2013). Often multiple *Ambrosiozyma* species occur together with platypodine beetles and other ambrosia fungi in the same gallery (Endoh et al., 2011; Yun et al., 2015). Their ecological role remains unclear, and their morphological uniformity prevented us from estimating their association fidelity as we did with filamentous ambrosia fungi.

Our study recovered a significant number of fungal species that appear to be new to science, despite the fact that they are mutualists of important insects in one of the most studied regions in the world. A much greater diversity of platypodine beetles – over 1400 species – resides in the tropics, but only a handful of species have been examined to identify their associated fungi. Furthermore, even the few studies that exist used methodology that is not always suitable for the recovery of the true symbionts. We strongly suggest that researchers use appropriate tools, including multiple types of media, and focus on the correct body part of the beetles to assure that this treasure trove of unexplored fungal diversity is reported accurately.

Acknowledgements

We would like to thank Adam Black and M. Patrick Griffith for their help and permission to collect beetles at the Montgomery Botanical Center (Miami, FL). Additional thanks to Thomas H. Atkinson (University of Texas, Austin, TX) for training the first author on platypodine identification. This project was funded by the United States Department of Agriculture-Animal and Plant Health Inspection Service Farm Bill Section 10007, the United States Department of Agriculture-Forest Service cooperative agreement 17-CA-11420004-100, the National Science Foundation (grant number: DEB 1556283), and the Florida Department of Agriculture-Division of Plant Industry.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.06.006.

References

Alfaro, R.I., Humble, L.M., Gonzalez, P., Villaverde, R., Allegro, G., 2007. The threat of the ambrosia beetle *Megaplatypus mutatus* (Chapuis) (= *Platypus mutatus* Chapuis) to world poplar resources. Forestry 80 (4), 471–479.

Alvidrez-Villarreal, R., Hernández-Castillo, F.D., Garcia-Martínez, O., Mendoza-Villarreal, R., Rodríguez-Herrera, R., Aguilar, C.N., 2012. Isolation and pathogenicity of fungi associated to ambrosia borer (*Euplatypus segnis*) found injuring pecan (*Carya illinoensis*) wood. Agric. Sci. 3 (3), 405–416.

Atkinson, T.H., 2004. Ambrosia Beetles, Platypus Spp. (Insecta: Coleoptera: Platypodidae). University of Florida IFAS Extension EENY174, DPI Entomology Circular, 321.

Atkinson, T.H., Peck, S.B., 1994. Annotated checklist of the bark and ambrosia beetles (Coleoptera: platypodidae and Scolytidae) of tropical southern Florida. Fla. Entomol. 77 (3), 313–329.

Bateman, C., Huang, Y.-T., Simmons, D.R., Kasson, M.T., Stanley, E.L., Hulcr, J., 2017. Ambrosia beetle *Premnobius cavipennis* (Scolytinae: Ipini) carries highly divergent ascomycotan ambrosia fungus, *Afroraffaelea ambrosiae* gen. nov. et sp. nov. (Ophiostomatales). Fungal Ecology 25, 41–49. https://doi.org/10.1016/j.funeco.2016.10.008.

Bateman, C., Kendra, P.E., Rabaglia, R., Hulcr, J., 2015. Fungal symbionts in three exotic ambrosia beetles, *Xylosandrus amputatus*, *Xyleborinus andrewesi*, and *Dryoxylon onoharaense* (Coleoptera: Curculionidae: Scolytinae: Xyleborini) in Florida. Symbiosis 66 (3), 141–148.

Bateman, C., Sigut, M., Skelton, J., Smith, K.E., Hulcr, J., 2016. Fungal associates of the *Xylosandrus compactus* (Coleoptera: Curculionidae, Scolytinae) are spatially segregated on the insect body. Environ. Entomol. 45 (4), 883–890. https://doi.org/10.1093/ee/nvw070.

Batra, L.R., 1963. Contributions to our knowledge of ambrosia fungi. II. *Endomycopsis fasciculata* nom. nov. (Ascomycetes). Am. J. Bot. 50 (5), 481–487.

Beaver, R., 1989. Insect–fungus relationships in the bark and ambrosia beetles. In: Wilding, N., Collins, N.M., Hammond, P.M., Webber, J.F. (Eds.), Insect–fungus Interactions. Academic Press, San Diego, CA, USA, pp. 121–143.

Beaver, R.A., 2013. The invasive neotropical ambrosia beetle *Euplatypus parallelus* (Fabricius, 1801) in the oriental region and its pest status (Coleoptera: Curculionidae, Platypodinae). Entomologist's Mon. Mag. 149, 143–154.

Belhoucine, L., Bouhraoua, R.T., Meijer, M., Houbraken, J., Harrak, M.J., Samson, R.A., Equihua-Martinez, A., Pujade-Villar, J., 2011. Mycobiota associated with *Platypus cylindrus* (Coleoptera: Curculionidae, platypodidae) in cork oak stands of North west Algeria, Africa. Afr. J. Microbiol. Res. 5 (25), 4411–4423. https://doi.org/10.5897/ajmr11.614.

Bellahirech, A., Inácio, M.L., Bonifácio, L., Nóbrega, F., Sousa, E., Ben Jamâa, M.L., 2014. Comparison of Fungi Associated with Platypus Cylindrus F. (Coleoptera: Platypodidae) in Tunisian and Portuguese Cork Oak Stands. IOBC/wprs Bulletin, 101 (Integrated Protection in Oak Forests), pp. 149–156.

Biedermann, P.H., Klepzig, K.D., Taborsky, M., Six, D.L., 2013. Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae, Scolytinae). FEMS Microbiol. Ecol. 83 (3), 711–723. https://doi.org/10.1111/1574-6941.12026.

Boa, E., Kirkendall, L.R., 2004. Strengthening National Capacity for Control of *Pterocarpus Indicus* Wilt Disease. Disease and Forest Protection: Final Technical Report, Sandragon Wilt Disease, Seychelles. TCP/SEY/0168, 25 pp.

Brockerhoff, E.G., Bain, J., Kimberley, M., Knížek, M., 2006. Interception frequency of exotic bark and ambrosia beetles (Coleoptera: Scolytinae) and relationship with establishment in New Zealand and worldwide. Can. J. For. Res. 36 (2), 289–298.

Brown, S.P., Rigdon-Huss, A.R., Jumpponen, A., 2014. Analyses of ITS and LSU gene regions provide congruent results on fungal community responses. Fungal Ecology 9, 65–68.

Bumrungsri, S., Beaver, R., Phongpaichit, S., Sittichaya, W., 2008. The infestation by an exotic ambrosia beetle, Euplatypus parallelus (F.) (Coleoptera: Curculionidae: Platypodinae) of Angsana trees (Pterocarpus indicus Willd.) in southern Thailand. Songklanakarin J. Sci. Technol. 30 (5), 579–582.

Ceriani-Nakamurakare, E., Slodowicz, M., Gonzalez-Audino, P., Dolinko, A., Carmarán, C., 2016. Mycobiota associated with the ambrosia beetle Megaplatypus mutatus: threat to poplar plantations. Forestry 89 (2), 191–200. https://doi.org/10.1093/forestry/cpw001.

Davis, T.S., 2015. The ecology of yeasts in the bark beetle holobiont: a century of research revisited. Microb. Ecol. 69 (4), 723–732. https://doi.org/10.1007/s00248-014-0479-1.

de Beer, Z.W., Wingfield, M.J., 2013. Emerging lineages in the Ophiostomatales. In: Seifert, K.A., de Beer, Z.W., Wingfield, M.J. (Eds.), Ophiostomatoid Fungi: Expanding Frontiers, Vol. 12. CBS Biodiversity Series. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, pp. 21–46.

Dreaden, T.J., Davis, J.M., De Beer, Z.W., Ploetz, R.C., Soltis, P.S., Wingfield, M.J., Smith, J.A., 2014. Phylogeny of ambrosia beetle symbionts in the genus Raffaelea. Fungal Biology 118 (12), 970–978.

Endoh, R., Suzuki, M., Okada, G., Takeuchi, Y., Futai, K., 2011. Fungus symbionts colonizing the galleries of the ambrosia beetle Platypus quercivorus. Microb. Ecol. 62 (1), 106–120. https://doi.org/10.1007/s00248-011-9838-3.

Farris, S., Funk, A., 1965. Repositories of symbiotic fungus in the ambrosia beetle *Platypus wilsoni* Swaine (Coleoptera: platypodidae). Can. Entomol. 97 (5), 527–532.

Faulds, W., 1977. A pathogenic fungus associated with *Platypus* attack on New Zealand *Nothofagus* species. N. Z. J. For. Sci. 7 (3), 384–396.

Fraedrich, S.W., Harrington, T.C., Rabaglia, R.J., Ulyshen, M.D., Mayfield III, A.E., Hanula, J.L., Eickwort, J.M., Miller, D.R., 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. Plant Dis. 92 (2), 215–224.

Gillett, C.P., Crampton-Platt, A., Timmermans, M.J., Jordal, B.H., Emerson, B.C., Vogler, A.P., 2014. Bulk de novo mitogenome assembly from pooled total DNA elucidates the phylogeny of weevils (Coleoptera: Curculionoidea). Mol. Biol. Evol. 31 (8), 2223–2237.

Gillett, C.P., Rubinoff, D., 2017. A second adventive species of pinhole-borer on the islands of Oahu and Hawaii (Coleoptera: Curculionidae: Platypodinae). Proc. Hawaii. Entomol. Soc. 49, 51–57.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52 (5), 696–704.

Gümüş, E.M., Ergün, A., 2015. Report of a pest risk analysis for *Platypus parallelus* (Fabricius, 1801) for Turkey. EPPO Bull. 45 (1), 112–118. https://doi.org/10.1111/epp.12190.

Harrington, T., 1981. Cycloheximide sensitivity as a taxonomic character in Ceratocystis. Mycologia 73 (6), 1123–1129.

Harrington, T.C., Aghayeva, D.N., Fraedrich, S.W., 2010. New combinations in Raffaelea, Ambrosiella, and Hyalorhinocladiella, and four new species from the redbay ambrosia beetle, Xyleborus glabratus. Mycotaxon 111 (1), 337–361.

Harrington, T.C., Yun, H.Y., Lu, S.-S., Goto, H., Aghayeva, D.N., Fraedrich, S.W., 2011. Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt pathogen, Raffaelea lauricola, originated in Asia. Mycologia 103 (5), 1028–1036.

Hubbard, H., 1896. Ambrosia Beetles. United States Department of Agriculture Yearbook, 1896, pp. 421–430.

Hulcr, J., Stelinski, L.L., 2017. The ambrosia symbiosis: from evolutionary ecology to practical management. Annu. Rev. Entomol. 62, 285–303. https://doi.org/10.1146/annurev-ento-031616-035105.

Inácio, M.L., Henriques, J., Lima, A., Sousa, E., 2012a. Ophiostomatoid fungi associated with cork oak mortality in Portugal. IOBC-WPRS Bull. 76, 89–92.

Inácio, M.L., Henriques, J., Nóbrega, F., Marcelino, J., Sousa, E., 2012b. Ophiostomatoid Fungi, a New Threat to Cork Oak Stands. Conference Paper: Present and Future of Cork Oak in Portugal Held by the Portuguese Society of Plant Physiology, pp. 87–92.

Ito, S., Murata, M., Yamada, T., 2003. Massive mortality of Fagaceous trees in Japan. Phytopathology 93 (6) supplement S102.

Jordal, B.H., 2014. Platypodinae. In: Leschen, R., Beutel, R. (Eds.), Handbook of Zoology. Arthropoda: Insecta: Coleoptera, Morphology and Systematics (Phytophaga), vol. 3. deGruyter Press, Berlin/New York, pp. 358–364.

Jordal, B.H., 2015. Molecular phylogeny and biogeography of the weevil subfamily Platypodinae reveals evolutionarily conserved range patterns. Mol. Phylogenet. Evol. 92, 294–307. https://doi.org/10.1016/j.ympev.2015.05.028.

Kasson, M.T., O'Donnell, K., Rooney, A.P., Sink, S., Ploetz, R.C., Ploetz, J.N., Konkol, J.L., Carrillo, D., Freeman, S., Mendel, Z., Smith, J.A., Black, A.W., Hulcr, J., Bateman, C., Stefkova, K., Campbell, P.R., Geering, A.D.W., Dann, E.K., Eskalen, A., Mohotti, K., Short, D.P.G., Aoki, T., Fenstermacher, K.A., Davis, D.D., Geiser, D.M., 2013. An inordinate fondness for *Fusarium*: phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol. 56, 147–157. https://doi.org/10.1016/j.fgb.2013.04.004.

Kile, G., Hall, M., 1988. Assessment of *Platypus subgranosus* as a vector of *Chalara australis*, causal agent of a vascular disease of *Nothofagus cunninghamii*. N. Z. J. For. Sci. 18 (2), 166–186.

Kim, K.-H., Choi, Y.-J., Seo, S.-T., Shin, H.-D., 2009. *Raffaelea quercus-mongolicae* sp. nov. associated with *Platypus koryoensis* on oak in Korea. Mycotaxon 110 (1), 189–197.

Kinuura, H., 2002. Relative dominance of the mold fungus, Raffaelea sp., in the mycangium and proventriculus in relation to adult stages of the oak platypodid beetle, Platypus quercivorus (Coleoptera; Platypodidae). J. For. Res. 7 (1), 7–12.

Kinuura, H., Kobayashi, M., 2006. Death of *Quercus crispula* by inoculation with adult *Platypus quercivorus* (Coleoptera: platypodidae). Appl. Entomol. Zool 41 (1), 123–128.

Kobayashi, M., Ueda, A., 2005. Wilt disease of Fagaceae trees caused by *Platypus quercivorus* (Murayama) (Coleoptera: platypodidae) and the associated fungus: aim is to clarify the damage factor. J. Jpn. For. Soc. 87 (5), 435–450.

Kostovcik, M., Bateman, C.C., Kolarik, M., Stelinski, L.L., Jordal, B.H., Hulcr, J., 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. ISME J. 9 (1), 126–138.

Kubono, T., Ito, S.-i., 2002. *Raffaelea quercivora* sp. nov. associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). Mycoscience 43 (3), 255–260. https://doi.org/10.1007/s102670200037.

Kurtzman, C.P., Robnett, C.J., 2013. Description of *Ambrosiozyma oregonensis* sp. nov., and reassignment of *Candida* species of the *Ambrosiozyma* clade to *Ambrosiozyma kashinagacola* f. a., comb. nov., *Ambrosiozyma llanquihuensis* f. a., comb. nov., *Ambrosiozyma maleeae* f. a., comb. nov., *Ambrosiozyma pseudovanderkliftii* f. a., comb. nov., and *Ambrosiozyma vanderkliftii* f. a., comb. nov. Int. J. Syst. Evol. Microbiol. 63 (10), 3877–3883.

Li, Y., Simmons, D.R., Bateman, C.C., Short, D.P., Kasson, M.T., Rabaglia, R.J., Hulcr, J., 2015. New fungus-insect symbiosis: culturing, molecular, and histological methods determine saprophytic polyporales mutualists of Ambrosiodmus ambrosia beetles. PLoS One 10 (9) e0137689.

Li, Y., Zhou, X., Lai, S., Yin, T., Ji, Y., Wang, J., Wang, S., Hulcr, J., 2018. First record of *Euplatypus parallelus* (Coleoptera: Curculionidae) in China. Fla. Entomol. 101 (1), 141–143. https://doi.org/10.1653/024.101.0127.

Linnakoski, R., Mahilainen, S., Harrington, A., Vanhanen, H., Eriksson, M., Mehtätalo, L., Pappinen, A., Wingfield, M., 2016. Seasonal succession of fungi associated with *Ips typographus* beetles and their phoretic mites in an outbreak region of Finland. PLoS One 11 (5) e0155622. https://doi.org/10.1371/journal.pone.0155622.

Massoumi Alamouti, S., Tsui, C.K.M., Breuil, C., 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. Mycological Research 113 (8), 822–835. https://doi.org/10.1016/j.mycres.2009.03.003.

Mayers, C.G., McNew, D.L., Harrington, T.C., Roeper, R.A., Fraedrich, S.W., Biedermann, P.H.W., Castrillo, L.A., Reed, S.E., 2015. Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. Fungal Biology 119 (11), 1075–1092.

McKenna, D.D., Sequeira, A.S., Marvaldi, A.E., Farrell, B.D., 2009. Temporal lags and overlap in the diversification of weevils and flowering plants. Proc. Natl. Acad. Sci. U.S.A. 106 (17), 7083–7088.

Musvuugwa, T., de Beer, Z.W., Duong, T.A., Dreyer, L.L., Oberlander, K.C., Roets, F., 2015. New species of Ophiostomatales from Scolytinae and Platypodinae beetles in the Cape floristic region, including the discovery of the sexual state of Raffaelea. Antonie Leeuwenhoek 108 (4), 933–950. https://doi.org/10.1007/s10482-015-0547-7.

Nakashima, T., 1975. Several types of the mycetangia found in platypodid ambrosia beetles (Coleoptera: platypodidae). Insecta matsumurana. New Series. J. Fac. Agric. Hokkaido Univ., Series Entomol. 7, 1–69.

Nakashima, T., 1979. Notes on the time when the new female adults of the ambrosia beetle Crossotarsus niponicus Blandford (Coleoptera: platypodidae) harvest their symbiotic fungi into their mycetangia. Insecta matsumurana. New Series. J. Fac. Agric. Hokkaido Univ., Series Entomol. 17, 1–19.

Nakashima, T., 1982. Function and location of mycetangia in ambrosia beetles. In: Akai, H., King, R.C., Morohoshi, S. (Eds.), The Ultrastructure and Functioning of Insect Cells, Proceedings of the International Conference Held in Sapporo, Japan, by the Society for Insect Cells, pp. 87–90.

Nobuchi, A., 1993. The Platypodidae (Coleoptera) found in Japan. Soc. Urban. Manag. 15 (1), 33–55.

Payton, I., 1989. Fungal (*Sporothrix*) induced mortality of kamahi (*Weinmannia racemosa*) after attack by pinhole borer (*Platypus* spp.). N. Z. J. Bot. 27 (3), 359–368.

Pereira da Silva, J.C., Putz, P., Carvalho Silveira, E.d., Flechtmann, C.A.H., 2013. Biological Aspects of Euplatypus Parallelus (F.)(Coleoptera, Curculionidae, Platypodinae) Attacking Hevea Brasiliensis (Willd. Ex a. Juss.) in São Paulo Northwest, Brazil, Proceedings of the 3rd Congresso Brasil. Heveicultura Held in Guarapari, Brazil, pp. 1–4.

Ploetz, R.C., Konkol, J.L., Narvaez, T., Duncan, R.E., Saucedo, R.J., Campbell, A., Mantilla, J., Carrillo, D., Kendra, P.E., 2017. Presence and prevalence of *Raffaelea lauricola*, cause of laurel wilt, in different species of ambrosia beetle in Florida, USA. J. Econ. Entomol. 110 (2), 347–354. https://doi.org/10.1093/jee/tow292.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25 (7), 1253–1256.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. Bioinformatics 19 (12), 1572–1574. https://doi.org/10.1093/bioinformatics/btg180.

Sanderson, F.R., King, F.Y., Anuar, S., Pheng, Y.C., Ho, O.K., 1996. A fusarium wilt (*Fusarium oxysporum*) of angsana (*Pterocarpus indicus*) in Singapore. Gard. Bull. (Singap.) 48 (1/2), 89–127.

Seifert, K.A., de Beer, Z.W., Wingfield, M.J., 2013. The Ophiostomatoid Fungi: Expanding Frontiers. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Simmons, D.R., de Beer, Z.W., Huang, Y.T., Bateman, C., Campbell, A.S., Dreaden, T.J., Li, Y., Ploetz, R.C., Black, A., Li, H.F., 2016. New *Raffaelea* species (Ophiostomatales) from the USA and Taiwan associated with ambrosia beetles and plant hosts. IMA Fungus 7 (2), 265–273.

Six, D.L., 2003. Bark beetle–fungus symbioses. In: Bourtzis, K., Miller, T.A. (Eds.), Insect Symbiosis, vol. 1. CRC Press, Washington, D.C., USA, pp. 97–114.

Skelton, J., Jusino, M.A., Li, Y., Bateman, C., Thai, P.H., Lindner, D.L., Hulcr, J., 2018. Detecting symbioses in complex communities: the fungal symbionts of bark and ambrosia beetles within Asian pines. Microb. Ecol. https://doi.org/10.1007/s00248-018-1154-8.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30 (9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

Tarno, H., Septia, E.D., Aini, L.Q., 2016. Microbial community associated with ambrosia beetle, Euplatypus parallelus on sonokembang, Pterocarpus indicus in Malang. AGRIVITA. J. Agric. Sci. 38 (3), 312–320.

Vanderpool, D., Bracewell, R.R., McCutcheon, J.P., 2017. Know your farmer: ancient origins and multiple independent domestications of ambrosia beetle fungal cultivars. Mol. Ecol. https://doi.org/10.1111/mec.14394 epub ahead of print.

Verrall, A.F., 1943. Fungi associated with certain ambrosia beetles. J. Agric. Res. 66 (3), 135–144.

Vilgalys, R., Hester, M., 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172 (8), 4238–4246.

Wood, S.L., 1993. Revision of the genera of platypodidae (Coleoptera). Great Basin Nat. 53 (3), 259–281.

Yun, Y.H., Suh, D.Y., Yoo, H.D., Oh, M.H., Kim, S.H., 2015. Yeast associated with the ambrosia beetle, *Platypus koryoensis*, the pest of oak trees in Korea. Mycobiology 43 (4), 458–466. https://doi.org/10.5941/MYCO.2015.43.4.458.

Zipfel, R.D., de Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multigene phylogenies define Ceratocystiopsis and grosmannia distinct from ophiostoma. Stud. Mycol. 55, 75–97.