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Fungal symbiont community and absence of detectable mycangia in invasive *Euplatypus* ambrosia beetles

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Abstract
Platypodinae ambrosia beetles depend on mutualistic fungi for food, and both partners cooperate in colonizing dead trees. The fungi are transported in specialized structures (mycangia), but the location of mycangia is unknown in many platypodine species. One species with elusive mycangia is *Euplatypus parallelus*, widespread in the Americas, and recently invasive worldwide. Drawing on knowledge about other ambrosia beetles, we predict that the mycangia may be either internal in the head, internal or external within the prothorax, or the symbiont is carried within the hindgut. We attempted detection using X-ray computed tomography, Fluorescence In Situ Hybridization and histology. For method validation and comparison we used *Euplatypus compositus*, a related species with pronotal mycangia. Despite routine isolation of the ambrosia fungi from both sexes, no consistent mycangia-like structures were found anywhere within *E. parallelus*. Both *Euplatypus* species yielded a diverse fungal community on different body parts, but the most consistent associate of both beetle species, and the most likely nutritional mutualist, is *Raffaelea xyleborini*. A notable discovery is that during dispersal in both species, females had their hindgut filled with a mass of tightly packed yeasts, mostly an unknown *Starmera* species. The function of this yeast cache is not known. Our results showed that both *Euplatypus* species are associated with the same fungus, but *E. parallelus* either does not have mycangia or we failed to locate them. This study adds to the growing evidence that Platypodinae beetles have coevolved with members of the genus *Raffaelea* and that they are promiscuous at the genus level.

Keywords Platypodinae · Fungal diversity · *Ophiostomatales* · Fungus transport · Mutualists

1 Introduction

Mutualisms between fungus-growing insects and their fungal partners are prime examples of reciprocal adaptations and co-evolution. The degree of mutual adaptation (physiological, behavioral, and morphological) usually mirrors the degree of dependency between the mutualists (Mueller and Gerardo 2002; Biedermann and Vega 2020). In obligately mutualistic systems, the insects may rely on the fungi as their nutritional source, and in turn fungi benefit from the association as a means for dispersal. Within this context, fungal partner(s) can be selected for adaptations that more optimally nourish their hosts, while the insects can evolve to transport the nutritional mutualists in specialized structures that help the fungal partners colonize new substrates (Mueller et al. 2005; Biedermann and Vega 2020). These reciprocal adaptations are thought to maintain the stability of

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the mutualism and help maintain or direct fidelity between partners.

Ambrosia beetles in the subfamilies Scolytinae and Platypodinae (Coleoptera: Curculionidae) transport fungi in specialized organs named mycangia or mycetangia (Li et al. 2019; Biedermann and Vega2020; Mayers et al. 2020, 2022). Some mycangia are lined with secretory glands that provide environments that facilitate long-term specific associations between the insect and the coevolved fungal mutualist (Skelton et al. 2019). Many beetle species introduce the fungi into galleries constructed in dead or freshly dead trees, occasionally in stressed but still living trees (Hulcr and Stelinski 2017). Unlike in other fungus-growing insects such as the attine ants and macrotermidide termites, where fungal farming appears to have occurred in single ancestral lineages, fungus-farming evolved in at least 11 different lineages within the Scolytinae (Johnson et al. 2018) and one time in Platypodinae (Jordal 2015). These various lineages of beetles that have coevolved with fungi are represented within at least six orders (Peris et al. 2021), and each system has evolved a separate type of mycangium (Mayers et al. 2022). Most beetle–fungal associations show narrow mutual fidelity, i.e. species-specific (Skelton et al. 2019). Only mutualisms involving the fungal genera *Raffaelea* and *Harringtonia* (*Ophiostomatales*, *Ascomycota*) appear to be looser, although current data are not sufficient to distinguish definite biological patterns from biased sampling (Kostovcik et al. 2015; Saucedo-Carabez et al. 2018; Mayers et al. 2022) or from horizontal transfer of fungi between beetles colonizing the same trees (Carrillo et al. 2014).

Platypodinae beetles comprise more than 1600 species (Wood and Bright Jr 1992), mostly occurring in the tropics. One of the largest platypodine genera is *Euplatypus*, which contains more than 50 species (Wood and Bright Jr 1992). Globally the most widespread and common platypodine is *Euplatypus parallelus* (Fabricius), a polyphagous species that has been recorded from more than 62 plant hosts in 20 families. Native to Central and South America (Silva et al. 2013; Rainho et al. 2021), this species has been introduced throughout Africa, Asia, and the Pacific Islands. This beetle colonizes dead or diseased trees, sometimes so rapidly that it is often confused to be the cause of tree death (Bumrungsri et al. 2008; Tarno et al. 2016; Li et al. 2018b; Tang et al. 2019; Lei et al. 2020).

In terms of symbiotic fungi, Platypodinae beetles are associated with a variety of *Harringtonia* and *Raffaelea* species (Li et al. 2018a; Araújo et al. 2022). However, despite the importance of these fungal genera for many ambrosia beetles, there are few studies on *E. parallelus*, or any other *Euplatypus* (Tarno et al. 2016; Li et al. 2018a; Araújo et al. 2022). Li et al. (2018a) postulated that an ophiostomatoid fungus named *Raffaelea* sp. 7 could be the dominant mutualist of *E. parallelus*. This was supported by: (i) high counts of colony-forming units (CFUs) from sampled beetles, and (ii) the same fungus occurring in beetles caught at light traps in both Miami, FL, USA and Hainan, China (Li et al. 2018a).

The most puzzling feature of *E. parallelus* is the apparent absence of mycangia. In fact, the location of these fungal-carrying structures is unknown in many of the 1,600 Platypodinae species (Kirkendall et al. 2015). In species where mycangia are known, females typically have external pits on the pronotum supported by internal glands opening in the pit lumen (Kirkendall et al. 2015; Mayers et al. 2022). This is the case in *Euplatypus compositus*, a relative of *E. parallelus*. Males in some Platypodinae species have small mycangia-like pits, which may be vestigial. In other platypodine genera such as *Crossotarsus*, females have mycangia located inside the head, with a likely opening into the oral cavity (Nakashima 1971), suggesting that researchers need to look for mycangia in areas beyond the obvious surface structures. Li et al. (2018a) suggested that mycangia inside the beetle body may be present in Platypodinae species that lack external cuticular structures (pits or sac-like mycangia), including *E. parallelus*. Building on these reports, we predict that *E. parallelus* carries mutualistic fungi in a structure that has not been discovered yet in this beetle species.

The beetle ecology does not offer straightforward clue as to why *E. compositus* has the pit mycangia, and *E. parallelus* does not. Both species colonize trunks and branches of freshly dead trees, both are highly polyphagous. *Euplatypus parallelus* is a tropical species, with some populations reaching out to the subtropics (Silva et al. 2013; Rainho et al. 2021). *E. compositus* is subtropical to temperate; the two species overlap in Central Florida. In terms of their mechanism of carrying symbiotic fungi, no difference is known.

Here we aimed to identify the mycangia of *E. parallelus*. We examined dispersing males and females, because these are expected to have mycangia loaded with the dominant mutualistic fungi (Li et al. 2019). We used a combination of X-ray computed tomography, fluorescence in situ hybridization (FISH), and histological examination to search for these putative structures. In addition, we aimed to determine fungal mutualists in both *E. parallelus* and *E. compositus* using quantitative culturing.

2 Methods

2.1 Beetle collection

Beetles were collected at sites in North Florida (Gainesville) and South Florida (Naples and Homestead), USA. Sampling in Gainesville and Naples occurred during the fall

in 2021 and in Homestead during spring in 2022. Light traps were set up using UV blacklight and 95% ethanol as the lure (Hulcr et al. 2022). A total of 13 *E. compositus* (six females and seven males) were collected in Gainesville at the light traps. In Naples, *E. parallelus* and *E. compositus* are sympatric. Light trapping in this locality resulted in 25 specimens of *E. parallelus* (12 females and 13 males) and 26 *E. compositus* (eight females and 18 males). In Homestead, light trapping resulted in 42 specimens of *E. parallelus* (15 females and 27 males). All specimens were transported live to the laboratory in glass jars containing moist paper towels. Beetle vouchers are kept in the UF Forest Entomology (UFFE) collection, School of Forest, Fisheries and Geomatics Sciences, University of Florida, Gainesville, USA. Specimens from Belize were collected with the logistic support and collaboration from the Friends of Conservation and Development under the permit from the Belize Forestry Department.

2.2 The search for putative mycangia of *Euplatypus parallelus*

We investigated different body parts of 23 specimens of *E. parallelus* using multiple methods, including histology, fluorescent in situ hybridization (FISH) protocols, and computerized tomopgraphy (CT) scanning approaches. For histology, we used seven females, five from the beetle pool collected in Naples and two females from the UFFE ethanol-preserved collection. The preserved specimens were collected by You Li at light traps in China in collaboration with Shanghai Academy of Landscape Architecture. Beetles were left at -20 °C for 5 min and then were embedded whole in Tissue-Tek® Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA, USA). The embedded samples were snap-frozen in isopentane cooled in a bath of liquid nitrogen, then immediately stored at -80 °C. Thin cross-Sect. (10 µm) were prepared in a CryoStat CM1950 (Leica, Wetzlar, Germany). Sections were collected on Superfrost Plus Fisherbrand™ microscope slides (Fisher Scientific, Hampton, NH, USA) and stained using hematoxylin and eosin or lactophenol aniline blue and examined using an Olympus® BX53 light microscope (Tokyo, Japan). As *E. compositus* has well-documented pit mycangia, we sectioned six *E. compositus* females collected in Naples for comparison.

Another four *E. parallelus* females from Naples were prepared for FISH and for periodic acid Schiff (PAS) staining. The PAS staining differentially stains polysaccharides in fungal cell walls and is used to determine the presence of fungi in tissues (Dring 1955). Beetles preserved in 95% ethanol were postfixed with 4% paraformaldehyde (PFA) in butanol 80% for 48 h. Wings, elytra and legs were removed for histological preparations. Head/prothorax and abdomen were separated and embedded separately in Technovit 8100 (Kulzer GmbH, Hanau, Germany). From each sample, two parallel section series were prepared in which the sections were always alternately distributed on two slides. This resulted in two series of sections, one of which was used for FISH and the other for PAS. Hybridization for FISH used a general probe for fungi (PF2-Cy5: 5'-CTCTGGCTTCACCCTATTC-3') (Kempf et al. 2000), and 4',6-diamidino-2-phenylindole (DAPI) for the beetle DNA counterstaining, following the method described by Kaltenpoth et al. (Kaltenpoth et al. 2014).

We used nanoCT scanning to examine eight *E. parallelus* specimens from the UFFE ethanol-preserved collection. Beetles were removed from ethanol and left to dry overnight, then processed whole and examined using a Versa 620 XRM high resolution X-ray microscope (ZEISS, Oberkochen, Germany). For µCT analysis, two individuals of *E. parallelus* specimens were used. The ethanol-preserved samples were post-fixed overnight with 4% PFA in 80% ethanol, dehydrated in absolute ethanol and contrasted with 1% iodine in absolute methanol for 24 h (Janke et al. 2022). Drying was performed with a Leica CPD300 automatic critical point dryer (Leica Microsystems, Wetzlar, Germany). All X-ray scans were made with a SkyScan 1272 microtomograph (Bruker, Kontrich, Belgium) and image analysis was performed with Dragonfly2020.2 [Object Research Systems (ORS) Inc., Montreal, Canada, 2020; software available at http://www.theobjects.com/dragonfly].

Finally, we also dissected hindguts from another four specimens of *E. parallelus* (two females and two males) from the beetle pool collected in Homestead and all of the 13 specimens of *E. compositus* (six females and seven males) from the beetle pool collected in Gainesville. Hindguts were dissected in 1x phosphate buffered saline (PBS) under an Olympus® SZX 16 stereomicroscope (Tokyo, Japan). Hindgut contents were examined using bright-field and phase-contrast microscopy on an Olympus® BX53 light microscope.

2.3 Fungal isolation

Fungal isolations were carried out on the day after the beetle collection. We used a total of 33 beetles for culturing the contents of the head and prothorax: 13 specimens from the pool collected in Naples (four females and five males of *E. parallelus*; two females and two males of *E. compositus*), and 20 specimens from the pool collected in Homestead (10 females and 10 males of *E. parallelus*). Head and prothorax of each specimen were separated with a sterile scalpel and processed separately. Hindguts of two additional *E. compositus* females from Gainesville and hindguts of all

the 20 specimens of *E. parallelus* from Homestead were also processed for fungal isolation following the procedure described above. Overall, the total number of samples used for culturing were: 33 heads, 33 prothoraces and 22 hindguts. No beetle part was surface sterilized prior to culturing.

Each beetle part was crushed separately in 1x PBS using a sterile plastic pestle, vortexed and serially diluted with 1x PBS (10, 100 and 1000 times). Suspensions were spread on Potato Dextrose Agar medium (PDA, BD Difco™, Sparks, MD, USA) supplemented with 100 mg L^{-1} of streptomycin sulphate (TCI America™, Portland, OR, USA) and 0.5 mg mL^{-1} of cycloheximide (Abcam, Waltham, MA, USA) according to Harrington et al. (Harrington et al. 2010) and Harrington (Harrington 1981). We used this semi-selective medium for the isolation of *Ophiostomatales* fungi and for the suppression of other fast-growing contaminant fungi (Harrington et al. 2010).

Plates were incubated at 25 °C for 15 days and examined daily for fungal growth. As soon as morphologically distinct colonies developed, the colony forming units (CFUs) were counted. Representative colonies of each morphotype were transferred to new PDA plates without antibiotics. These subculture plates were incubated under the same conditions as above. Single-conidium cultures were obtained by preparing conidia suspensions in distilled water. Serial dilutions of the suspension were surface spread on PDA. After incubation, single colonies were transferred to new PDA plates. Single-conidium cultures of each fungal isolate were stored in 10% glycerol at − 80 °C at the UF Forest Entomology Laboratory slant vial collection (UFFEsv). Only pure cultures were used for DNA sequencing. Raw data on isolated fungi, total beetles collected, beetle parts and sex are compiled in Supplemental Table S1.

2.4 Fungal identification

Morphotypes were tentatively identified by morphology and subsequently assigned via sequencing of genomic markers as detailed below. For colony characteristics and visualization of microscopic asexual structures, wet mounts were used from cultures grown on PDA for five days at 25 °C. For DNA sequencing, genomic DNA was extracted from fungal cultures as described in Li et al. (2018a). Briefly, hyphae or yeast cells were scraped from the colony of five day-old cultures using a sterile scalpel. This fresh fungal material was added to microtubes containing 20 µL of Extract-N-Amp™ Plant Tissue PCR Kits (Sigma-Aldrich, St. Louis, MO, USA). Samples were vortexed for 1 min and incubated in a thermocycler at 96 °C for 30 min. After spinning down samples, 20 µL of 3% Bovine Serum Albumin (BSA, Thermo Fisher Scientific, Waltham, MA, USA) was added to tubes. Samples were vortexed for 20 s and then spun at 6,000 RPM for 10 s. A total of 20 µL of supernatant was removed and stored at -20 °C prior to PCR amplification.

Three genomic markers were examined: the 28S large subunit (LSU), the ß-tubulin gene, and the internal transcribed spacer (ITS) region. The following primer pairs were used: LROR 5'-ACCCGCTGAACTTAAGC)/LR5 (5'-TCCTGAGGGAAACTTCG) for LSU (Vilgalys and Hester 1990; Rehner and Samuels 1994), Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC)/Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC) for ß-tubulin (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA)/ITS4 (5'-TCCTCCGCTTATTGATATGC) for ITS (White et al. 1990; Gardes and Bruns 1993). PCR conditions were as follows: LSU (94 °C for 3 min, 30 cycles of 94 °C for 1 min, 50 °C for 45s, and 72 °C for 1 min), ß-tubulin (95 °C for 3 min, 35 cycles of 95 °C for 30s, 57 °C for 45s, and 72 °C for 40s, final extension at 72 °C for 8 min), and ITS (94 °C for 3 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min). PCR was performed in a final volume of 25 µL using 12.5 µL of Premix Taq™ (Takara Bio Inc., Kusatsu, Shiga), 1 µL of each of the 10 mM primers (Integrated DNA Technologies, Inc., Coralville, IA), 1 µL of 5% dimethyl sulfoxide (Thermo Fisher Scientific, Waltham, MA, USA), 1 µL of 10 mg mL^{-1} BSA, 1 µL of DNA template and 7.5 µL of ultrapure water. Amplicons were visualized in 1% agarose gels after electrophoresis in Tris-acetic acid buffer using SYBR™ Green Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA).

Successful PCR amplicons were sent for Sanger sequencing at Eurofins Genomics LCC (Louisville, KY, USA) using the same primers used in amplification. Bidirectional sequences were assembled and inspected for quality in Geneious 9.1.8 (https://www.geneious.com). Sequences were deposited in NCBI-GenBank under accessions listed in Supplemental Table S2. ITS sequences were generated as reference for barcoding purposes only but were not used in the phylogenetic analyses. We performed BLAST queries against the curated RefSeq database on GenBank for fungi. We limited our search to type material only by selecting "Sequences from type material" to guide our preliminary identifications (results in Supplemental Table S1). A combination of morphology and phylogenetic analyses supported the final identification of fungal isolates (Supplemental Table S1).

2.5 Phylogenetic analyses

The sequences obtained in this study were first placed within the context of the broader *Ophiostomatales* phylogeny using LSU sequences deposited in GenBank and derived from studies on bark and ambrosia beetles and their

plant hosts (de Beer et al. 2022; Simmons et al. 2016 and references there in). The final dataset was composed of 234 sequences with a total length of 967 bp.

Second, to place isolates within the genera *Dryadomyces*, *Harringtonia*, or *Raffaelea*, we used a concatenated dataset with two partitions (LSU and ß-tubulin). We assembled the data set with 180 taxa (1206 bp in length) obtained from beetles and their associated host plants (Funk 1970; Ohtaka et al. 2006; Yamaoka et al. 2009; Massoumi Alamouti et al. 2009; Harrington et al. 2010; Dreaden et al. 2014; Musvuugwa et al. 2015; Simmons et al. 2016; Procter et al. 2020; de Beer et al. 2022). In addition to sequences from fungi obtained in this study, we included additional sequences of *Raffaelea*, *Dryadomyces* and *Harringtonia* species isolated from *E. parallelus* collected from Brazil (4 isolates), Belize (1) and the US (13) in previous unpublished studies. Cultures of these isolates are deposited at the Laboratory of Fungal Ecology and Systematics (LESF, São Paulo State University, Rio Claro, Brazil) and in the UFFEsv collection.

Third, for genera in the *Saccharomycetales*, we assembled partial LSU sequences deposited in GenBank, mostly from reference strains and from previous phylogenies of the respective genera (Yamada et al. 1996; Kurtzman and Robnett 2013; Naumov et al. 2017; Moreira et al. 2020). Specifically, we assembled datasets for the genera *Ambrosiozyma* (47 taxa, 915 bp in length), *Ogatea* (24 taxa, 905 bp in length), *Saccharomycopsis* (28 taxa, 608 bp in length), and *Starmera* (40 taxa, 935 in length).

Sequences were aligned in MAFFT using the auto selection strategy (Katoh and Standley 2013). For ß-tubulin, introns were excluded before alignment following Simmons et al. (2016). For Maximum likelihood-like (ML-L) analysis, nucleotide substitutions models were calculated using Bayesian Information Criteria (BIC) implemented in IQ-TREE v. 2.0.7. (Kalyaanamoorthy et al. 2017): TIM3 + F + I + G4 for the *Ophiostomatales* tree; TIM2 + F + I + G4 (partition 1: ß-tubulin) and TN + F + R3 (partition 2: LSU) for the *Raffaelea*, *Dryadomyces* and *Harringtonia* tree; for *Saccharomycetales*: TIM3 + F + R2 for the *Ambrosiozyma* tree; TPM3u + F + I + G4 for the *Ogatea* tree; TIM3 + F + I + G4 for the *Saccharomycopsis* tree; and TIM3 + F + I + G4 for the *Starmera* tree. Phylogenetic trees were reconstructed with: ML-L with ultrafast bootstrap approximation in IQTREE2 (Nguyen et al. 2015), using 1000 replicates of ultrafast bootstrap and 1000 iterations. Bayesian Inference was carried out in ExaBayes (Aberer et al. 2014). We set two separate runs with 10.000 generations of the Markov Chain Monte Carlo which were enough to reach convergence (standard deviation of split frequencies was below 0.01). We used the GTR model for each partition independently for all alignments. The first 25% of trees were discarded as burn in for final tree reconstruction. Trees were edited in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and then in Adobe Illustrator 2020.

3 Results

3.1 *Euplatypus compositus*, but not *E. parallelus*, transport fungi in evident mycangia

Several morphological structures of the beetle containing fungi were found in *E. parallelus*, but none was present consistently in all specimens. In one of seven *E. parallelus* females sectioned for histology, we found yeast-like cells internally at the base of the maxillae (Fig. 1a). Additionally, in one of seven *E. parallelus* females, a clump of fungal conidia was present in a crevice at the base of the prothorax, beneath a row of setae (Fig. 1b). Micro-CT scanning of two specimens showed accumulation of fibrous material in the ventral side of the head (Fig. 2a and b). Reconstruction analyses of the images suggested a putative cavity that had no direct connection to the digestive tract (Fig. 2c and e). A potential opening to this cavity was located below the mandibles, that was further delimited by the maxillae (lateral) and the labium (ventral, Fig. 2d). The mouth opening was located at the level of the mandibles and above the opening of the cavity (Fig. 2e). PAS staining confirmed that the

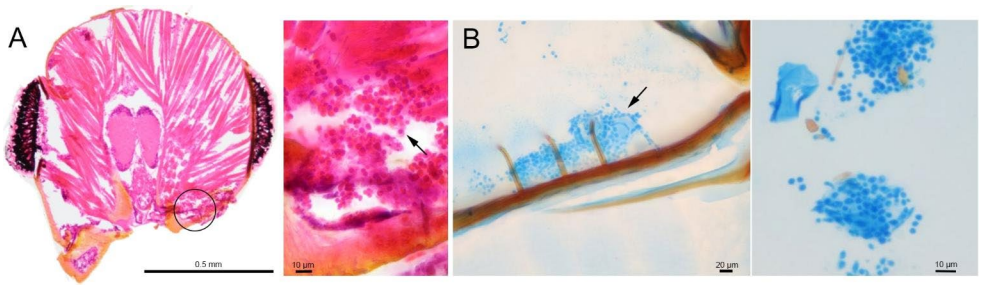


Fig. 1 *Euplatypus parallelus* dispersing females. **A** Cross section of a head stained with hematoxylin and eosin. The base of the missing mandible is highlighted where putative fungal cells were observed. Putative fungal cells (arrow). **B** Cross section of the dorsal part at the base of the prothorax, where fungal cells are attached to external setae (arrow). Inset: fungal cells detached from setae (lactophenol aniline blue staining). These images are derived from representative slides, but all layers throughout the head and pronotum were examined

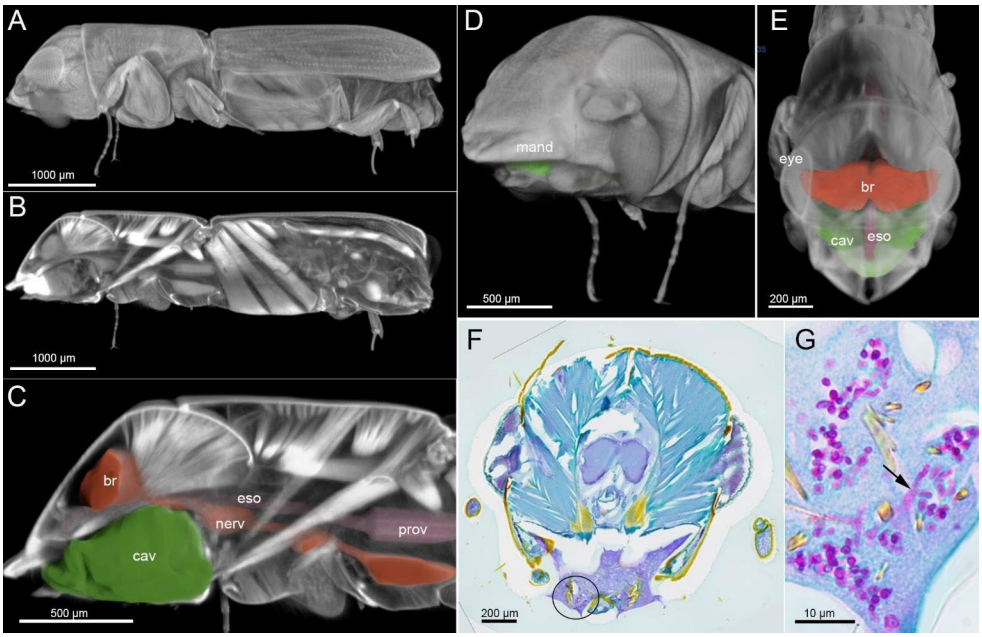


Fig. 2 *Euplatypus parallelus* female examined by nano-CT scanning. **A** Surface of the whole beetle. **B** Longitudinal section of the whole beetle. **C** Reconstruction of the head and prothorax showing internal organs and the cavity (in green). **D** Opening of the cavity located under the mandibles which is further delimited by the maxillae. **E** Esophagus apparently overlaying the cavity with no connection between the two. The opening of the mouth is at the level of the mandibles above the opening of the cavity. **F** Cross section of the head stained with Periodic acid Schiff (PAS). Black circle denotes the region in the cavity where yeast-like cells were observed. **G** Close image of the putative fungal cells (arrow). br brain, cav cavity, nerv nerve, eso esophagus, prov proventriculus, mand mandibles

interior of the cavity was filled with a fibrous mass (Fig. 2f) that was not of fungal origin and contained relatively small accumulations of yeast-like cells (Fig. 2g).

FISH analyses using general fungal primers failed to detect any signals of hybridization in any of sections of the head or prothorax in a set of four females examined (Fig. 3a and b). Nano and micro-CT scanning similarly failed to reveal any evidence of fungal masses in the prothorax or on the surface of pronotum in all eight specimens examined for this purpose (Online Resource Fig. S1). FISH signals indicated the presence of fungal cells in all tested hindguts (Fig. 3c and d), with yeast-like cells were observed in the posterior portion of the midgut and in the hindgut of all seven females sectioned.

For comparative analyses and to ensure methodological validity, in the control species *E. compositus*, as expected, histological sections showed evident dense masses of fungal cells in the pit mycangia in all six females (Online Resource Fig. S2). In addition, the sections also clearly showed yeast-like cells in the hindgut (Online Resource Fig. S2). As both beetle species showed the presence of fungi in the hindgut, we dissected additional males and females of *E. compositus* and *E. parallelus*. Hindguts of both beetle species contained a yellow mass (Fig. 4a and d). However, close examination revealed that in females, the yellow mass was a tightly packed clump of yeast-like cells, filling up the hindgut lumen (in all dissected specimens: six of *E. compositus* females – Fig. 4b and two *E. parallelus* females – Fig. 4c). In males, the hindgut was narrower, its content was sparser and inconsistent, and appeared to be composed of diet and bacterial cells of variable morphologies with no tightly packed clumps of yeast-like cells (in all seven *E. compositus* males, Fig. 4e and two *E. parallelus* males, Fig. 4f).

3.2 Diverse fungi found in the head and prothorax of *Euplatypus* species from Florida

Although no compelling evidence was found for mycangia in *E. parallelus* in contrast to the conspicuous mycangia of *E. compositus*, we obtained 132 fungal isolates in pure culture from *E. parallelus* (14 females and 15 males) and 24 fungal isolates from *E. compositus* (two females and two males) caught at light traps. From the fungal isolate/body part screening, no single body part consistently yield high numbers of fungal colony-forming units (CFU) that could suggest the potential location of the mycangia in *E. parallelus* (Table 1). Instead fungi were cultured in approximately equal abundance from both the head and the prothorax (Table 1). Morphological examinations combined with DNA sequencing, allowed for the discrimination of the isolates into 19 fungal taxa (Table 1) belonging to *Ophiostomatales* (genera *Ceratocystiopsis*, *Dryadomyces*, *Esteya*, *Harringtonia*, *Raffaelea* and *Sporothrix*; Online Resource Fig. S3) and yeasts in the *Saccharomycetales* (*Ambrosiozyma*,

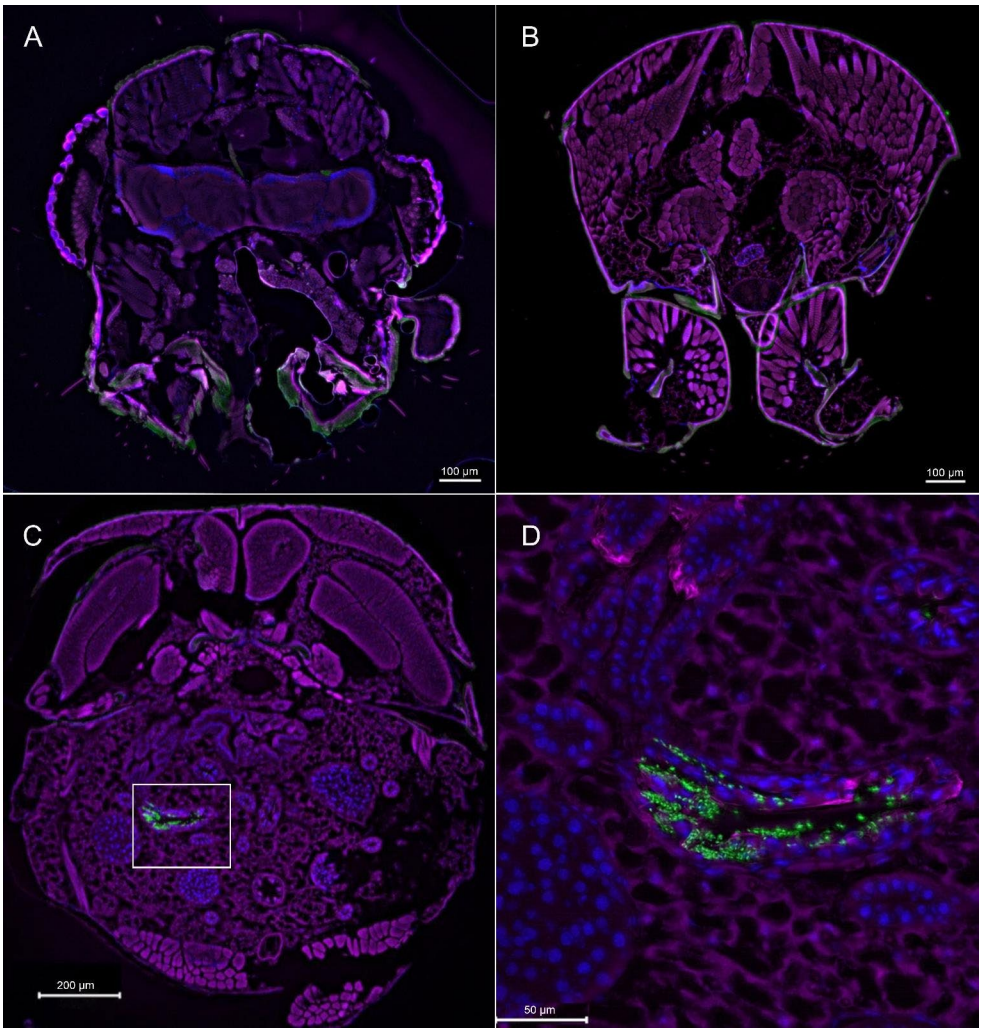


Fig. 3 Sections of *Euplatypus parallelus* dispersing females hybridized with PF2-Cy5 probe (universal probe for fungi). **A** and **B** Cross sections of the head and prothorax with no detected signals of fungal cells. **C** and **D** Cross section of the anterior portion of the abdomen showing internal parts of the hindgut, showing fluorescent signals of fungi. These images are derived from representative slides, but all sections throughout the head and pronotum were examined

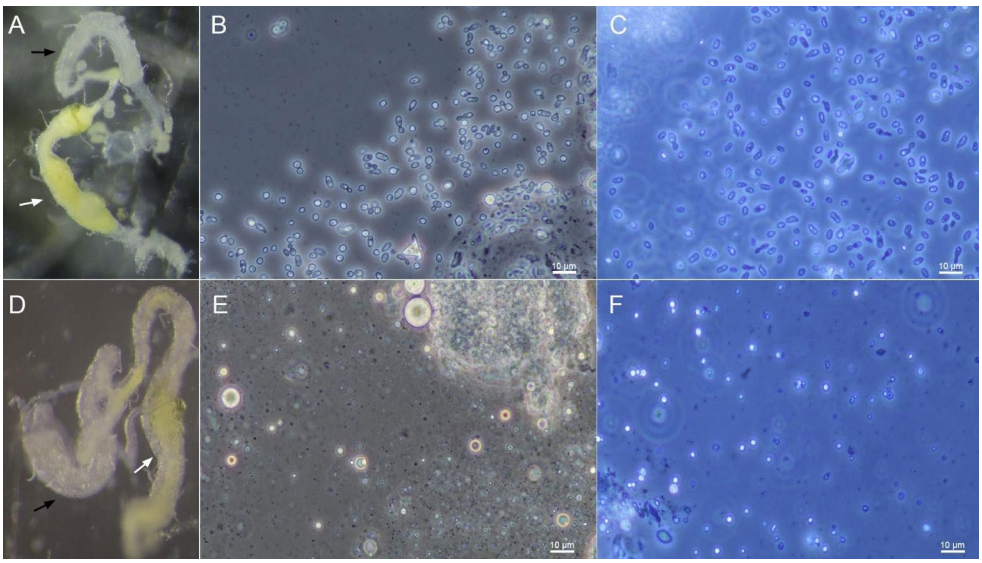


Fig. 4 Dissected hindguts (white arrows) and midguts (black arrows) of dispersing *Euplatypus compositus* and *Euplatypus parallelus* beetles. **A** Hindgut of a *E. compositus* female showing the yellow mass of dietary contents. Microscopic examination revealed abundant yeast cells in the hindguts of all females of **B** *E. compositus* (six females) and **C** *E. parallelus* (two females). **D** Hindgut of a *E. compositus* male also showing the yellow mass. Microscopic examination did not reveal abundant yeast cells in **E** *E. compositus* (seven males) and **F** *E. parallelus* (two males)

Table 1 Fungi obtained by in vitro cultivation from body parts of two *Euplatypus* species caught with light traps

Fungi	# isolates[1]	Colony-forming units (mean ± standard error)					
		E. compositus (n = 4)			*E. parallelus* (n = 29)		
		Head	Prothorax	Prevalence[2]	Head	Prothorax	Prevalence[2]
Saccharomycetales							
Ambrosiozyma sp.	31		85 ± 5	50	186 ± 69	92 ± 28	55.2
Candida sp.	1				730		3.4
Diapodascopsis sp.	1					170	3.4
Ogataea sp. 1	1				10		3.4
Ogataea sp. 2	5				573 ± 462	25 ± 15	13.8
Saccharomycopsis sp.	7				43 ± 28	30 ± 4	24.1
Starmera dryadoides	2				140 ± 30		6.9
Starmera sp.	9				55 ± 30	58 ± 26	20.7
Ophiostomatales							
Ceratocystiopsis lunata	15	350	990 ± 484	75	743 ± 508	611 ± 429	27.5
Dryadomyces sp.	2					100	3.4
Esteya floridana	2	200	100	25			
Harringtonia arthroconidialis	14				105 ± 45	89 ± 39	41.3
Harringtonia chlamydospora	1		10	25			
Harringtonia sp.	1					10	3.4
Raffaelea xyleborini	48	75 ± 30	1170 ± 363	75	152 ± 49	351 ± 125	72.4
Raffaelea sp. 1 (*scolytodis*-clade)	5				15 ± 5	86 ± 29	13.8
Raffaelea sp. 2 (homestead-clade)	5				75 ± 15	33 ± 6	10.3
Raffaelea sp. 3 (*subalba*-clade)	5	470	842 ± 419	75			
Sporothrix sp.	1				50		3.4
Total	156						

[1] Total number of isolates obtained after subculturing morphotypes from isolation plates

[2] Percentage of beetle individuals carrying fungus relative to the total number of individuals sampled for each species: *E. compositus* (4 individuals) and *E. parallelus* (29 individuals)

Candida, *Diapodascopsis*, *Ogataea*, *Saccharomycopsis*, and *Starmera*; Online Resource Fig. S4-S7). *Ceratocystiopsis lunata*, *Raffaelea xyleborini*, and *Raffaelea* sp. 3 were prevalent in *E. compositus* and occurred in 75% of the specimens (Table 1). In *E. parallelus*, *R. xyleborini* was the prevalent fungus and was found in 72.4% of the specimens (Table 1) in relatively high CFU numbers in both sexes from both the head and prothorax samples (Online Resource Fig. S8).

The ophiostomatalean fungi grouped into six phylogenetic clades, together with fungal species reported from other bark and ambrosia beetles (Online Resource Fig. S3). Isolates identified as *C. lunata* clustered in a well-supported clade (98% ML bootstrap support, but with PP below 0.7) along with isolates from various platypodine genera (including *Euplatypus*) and from *Xylosandrus crassiusculus* (Scolytinae, Online Resource Fig. S3). Isolate UFFEsv 18,289 was identified as *Esteya floridana* and found in low CFU counts in *E. compositus* (Table 1). Likewise, this isolate clustered with others obtained from *Myoplatypus flavicornis* (Platypodinae). Isolate UFFEsv 18,291 grouped in the *Dryadomyces* clade, along with isolate UFFEsv 17,601, both of which were previously reported from *E. parallelus* in Florida (Fig. 5). *Dryadomyces* sp. 18,291 was isolated from the prothorax of *E. parallelus* in low CFU counts and appears to be an undescribed species (Table 1). As for the isolates that grouped in the *Harringtonia* clade, they belong to one potentially undescribed (UFFEsv 18,452) and two recently described species: *H. arthroconidialis* (here found in the head and prothorax of *E. parallelus* only) and *H. chlamydospora* (here found in the head of *E. compositus*, Fig. 5), although both in low CFU counts (Table 1).

Phylogenetic analysis also showed two isolates (LESF 1117 and LESF 1120) obtained from *E. parallelus* in Brazil with uncertain position within the *Ophiostomatales*. In the order-level analysis (Online Resource Fig. S3) they grouped as sister of the clade formed by *Harringtonia* and *Raffaelea* with high ML bootstrap and PP support. In contrast, in the genus level-analysis these isolates grouped as sister of *Esteya* and *Dryadomyces*, also with high ML bootstrap and PP support (Fig. 5). The monophyly as well as the uncertain position between the analyses and datasets used suggest that the two isolates represent a new lineage in the *Ophiostomatales*.

3.3 Hindguts dominated by yeasts

We obtained four fungal taxa from hindgut contents of both beetle species: *Ambrosiozyma* sp. (3 isolates), *C. lunata* (1), *R. xyleborini* (1) and *Starmera* sp. (15). These taxa were the same fungi that were also recovered from the head and prothorax samples. However, these fungi were observed in low prevalence and in low CFU counts in the hindgut, except for the yeast *Starmera* sp. (Fig. 4). We detected *Starmera* sp. in extremely high CFU counts in nine out of 10 *E. parallelus* females and in both *E. compositus* females examined (Fig. 4). Phylogenetic analysis showed it is closely related to

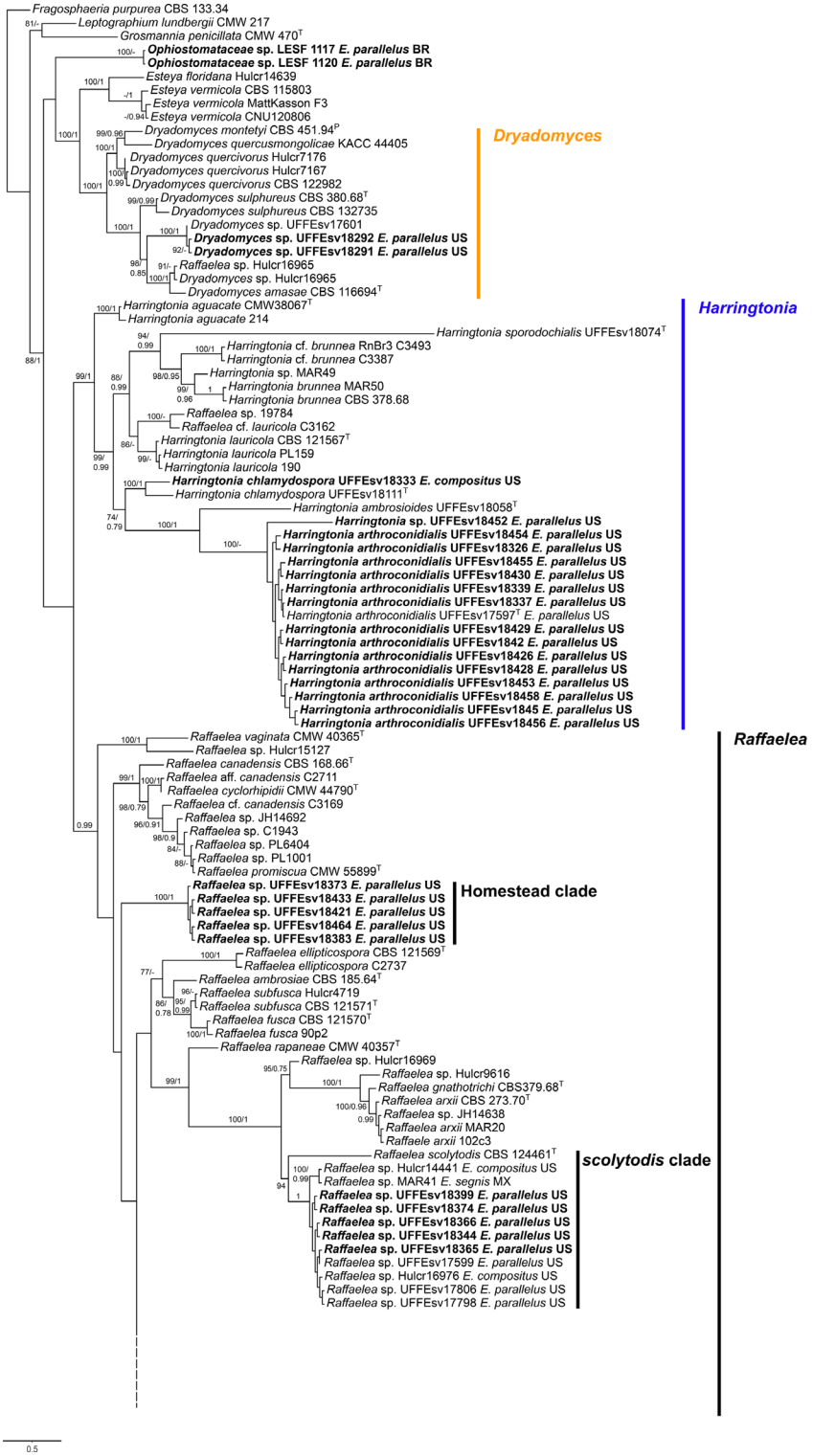
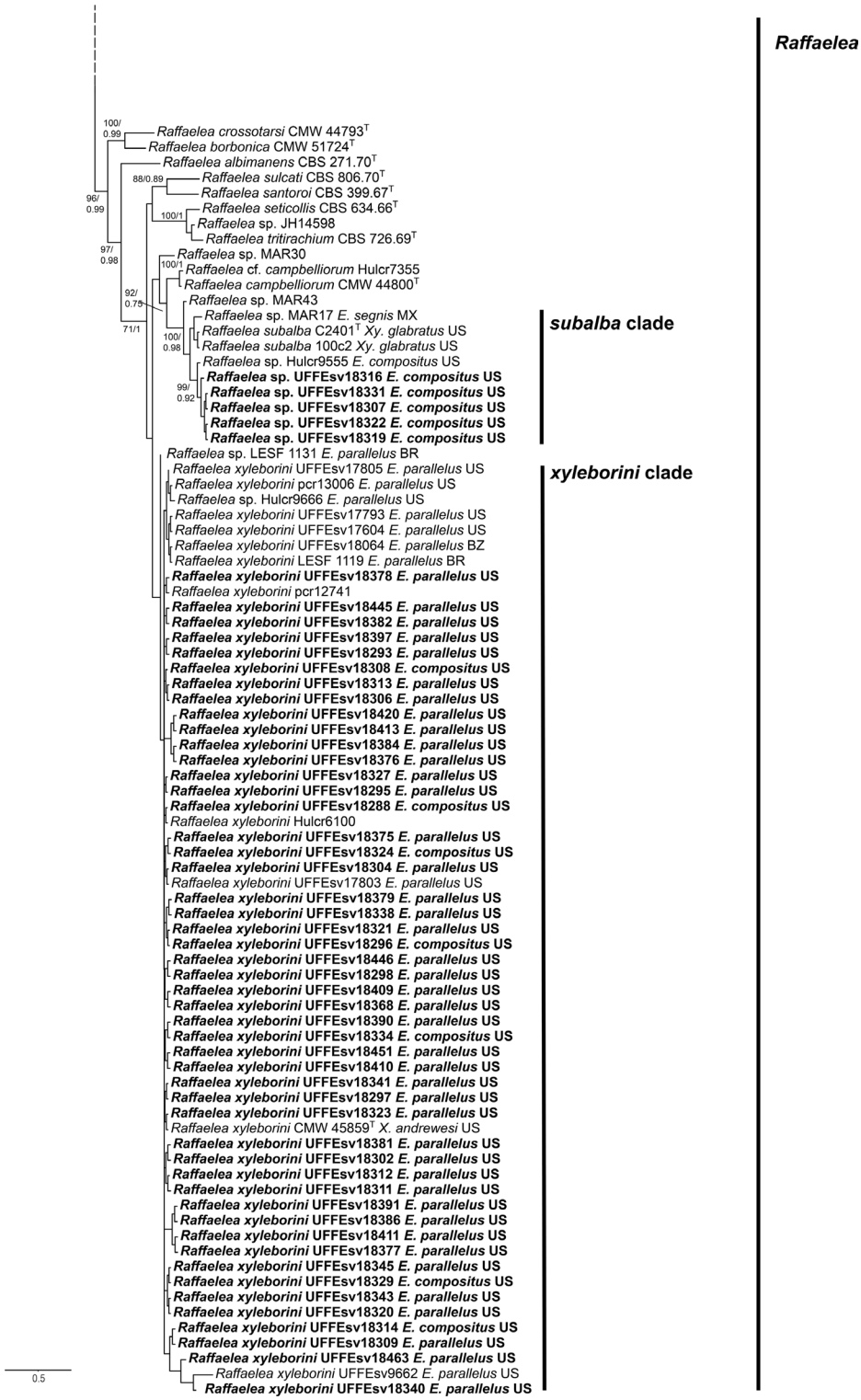


Fig. 5 Phylogenetic tree of taxa in the genera *Dryadomyces*, *Harringtonia* and *Raffaelea* based on ß-tubulin and partial large subunit (LSU) sequences. Names in bold denote fungi isolated from *Euplatypus compositus* and *E. parallelus* in this study, followed by the UFFE slant vial collection of the Forest Entomology Lab, University of Florida, Gainesville, USA, beetle origin and country (see also Tables S1 and S2). Other taxa names are followed by culture collection accessions. The tree shown is the consensus tree obtained by Bayesian Inference (BI), but analysis was also carried out under Maximum Likelihood-like criterium. Numbers on branches correspond to bootstrap support and posterior probabilities, respectively. The dataset contains a total of 180 taxa with total length of 1206 bp (LSU: 180 sequences and 963 bp in length; ß-tubulin: 82 sequences and 243 bp in length). *Fragosphaeria purpurea* CBS 133.34 as used as outgroup. BR: Brazil, BZ: Belize, MX: Mexico, US: United States. T: ex-type, P: ex-paratype

Fig. 5 (Continued)



Starmera dryadoides (Fig. S5), but LSU sequences showed only 94% similarity (Online Resource Table S1), suggesting that our isolate may represent an undescribed species.

3.4 *Raffaelea* phylogenetic diversity in *Euplatypus*

Euplatypus compositus and *E. parallelus* transport and feed on various *Raffaelea* species, including undescribed taxa (Li et al. 2018a). Phylogenetic analysis revealed *Raffaelea* isolates grouped in four clades that also contained isolates known from other Scolytinae and Platypodinae beetles and from different localities (Fig. 5). The dominant clade was determined as a *R. xyleborini*-clade composed of 49 isolates mostly from *E. parallelus*, but also from *E. compositus*, including the type specimen originally isolated from the Scolytine *Xyleborinus andrewesi* (Fig. 5). The morphological characters of our isolates also support the identification as *R. xyleborini* (data not shown). Fungi isolated from *E. parallelus* in previous collections in Belize, Brazil, Mexico, and the United States also clustered in this clade, indicating how widespread *R. xyleborini* is across the Americas (Fig. 5). We observed *R. xyleborini* in 24 out of 33 individuals sampled (72.7%), found in both the head and prothorax, with high CFU counts in the prothorax (Table 1). *Raffaelea xyleborini* was found in 71.4% and 73.1% of the total males (n = 7) and females (n = 26) of both beetle species (Online Resource Fig. S8).

The *subalba*-clade was comprised of five isolates obtained in the present study (named *Raffaelea* sp. 3, Table 1) and the isolate *Raffaelea* sp. Hulcr9555 from a previous study (Fig. 5). This group forms a monophyletic clade with *R. subalba* and contains isolates derived only from *E. compositus*. Fungi in this clade were found in both the head and prothorax of *E. compositus*, but were particularly abundant in the prothorax (Table 1). The *scolytodis*-clade was comprised of five isolates obtained in this study of an undescribed *Raffaelea* species (named *Raffaelea* sp. 1, Table 1) that is closely related to *Raffaelea scolytodis* (94% of ML boostrap support, Fig. 5). Curiously, all isolates of this species were derived only from *Euplatypus* species: *E. compositus* (Florida), *E. parallelus* (Florida), and *E. segnis* (Mexico, Fig. 5). Finally, another set of five isolates clustered in *Raffaelea* but as a separate clade (here named as Homestead clade), that contained *Raffaelea* sp. 2 (Fig. 5; Table 1). This clade comprised only isolates obtained from *E. parallelus* collected in Homestead, FL and was isolated from three different beetle specimens (Online Resource Table S1).

4 Discussion

Although ambrosia beetles have specialized structures to transport fungi, the precise location of the mycangia remains unknown for many Platypodinae species (Kirkendall et al. 2015; Mayers et al. 2022). Here we confirmed that *E. compositus* carries fungal mutualists in pit mycangia located on the pronotum. However, in *E. parallelus* fungal masses were found inconsistently, within at least three body parts: a cavity in the head capsule, a setose furrow on the base of the pronotum, and the hindgut; no clear mycangial structure was identified. We cultured mutualistic fungi in high abundances from both the head and the prothorax, but we failed to detect mycangia *sensu stricto*, i.e., specialized structures with secretory glands consistently housing fungi (Six 2012; Hulcr and Stelinski 2017). Our data indicated that *E. compositus* and *E. parallelus* collected during dispersal carry multiple described and undescribed ophiostomatalean fungi, but *R. xyleborini* was consistently present in dispersing females in the highest abundance and appeared to be the most prevalent fungus associated with these beetles. This same fungus was also found in *E. parallelus* collected in Brazil and Belize (this study), suggesting that it is the dominant symbiont of this beetle in its native range.

Two main types of mycangia have been reported for Platypodinae: pits on the pronotum, as in *Platypus* and *Oxoplatypus*, and internal mycangia in the head, as in *Crossotarsus* (Nakashima 1971; Bickerstaff & Hulcr, unpublished data). The selection pressure on the evolution and conservation of these organs comes not just from the need to transport the fungi, but also to facilitate the selection of specific, vertically inherited symbionts (Skelton et al. 2019; Mayers et al. 2022). *Euplatypus compositus* has the pronotal pit mycangium; there no reports of additional structures that may serve as the organ for symbiont selection. We could not detect any analogous pit mycangia in the related and much more widespread *E. parallelus*. A recent study by Tarno et al. (2016) assumed that pit mycangia are present on the pronotum but they did not conduct detailed studies of the beetles. Our CT scans did not reveal any evidence of fungal cells or fungal masses forming a characteristic structure that resembles a mycangium. Similarly, cryosections and FISH assays failed to consistently detect fungi in the head and pronotum, despite some putative structures that resembled fungal cells, however, these were not found on all beetles, and therefore likely represent collection artifacts. The lumps of yeast-like cells found in one specimen internally at the base of maxillae and in one specimen in a cavity under the mouth remain the most promising candidates as the transport mechanism for *E. parallelus* to vector fungi. As these yeast-like cells were detected in a few individuals, cannot be used as definitive evidence, and further research is needed. External structures such setal brushes at the base of the pronotum are routinely associated with mycangia, but it is unclear how such non-glandular, non-selective, exposed surfaces alone (in absence of a mycangium) would facilitate the sensitive process of symbiont selection.

How does *E. parallelus* transport its symbiont(s)? Several possibilities exist; first, and most likely, a mycangium exists but our methods did not detect it. In some fungus-associated beetles, the mycangium is minute, occurs within an unexpected body part, and is difficult to discover (Francke-Grosmann 1967). Alternatively, *E. parallelus* may have secondarily lost its mycangium and rely on use a passive mechanism of symbiont transfer, either vertical on its cuticle, or horizontal, from other ambrosia beetles. Neither is very likely given the narrow specificity to *R. xyleborini*, which is ubiquitous in this species and rare or absent in other sympatric ambrosia beetles.

The hindguts of females of both *E. parallelus* and *E. compositus* are packed with yeast cells, particularly *Starmera* sp. in *E. parallelus*. According to the phylogenetic analyses of genomic marker loci, the yeast isolates from *E. parallelus* are closely related to *S. dryadoides* but may represent an undescribed species. Species in the genus *Starmera* usually inhabit plant tissues (Moreira et al. 2020), which may explain the occurrence of this yeast in *E. parallelus*. However, this does not explain the high abundance found in the hindguts of several individual beetles. Yeasts have been reported from ambrosia beetles for a long time but are somewhat overlooked by researchers that usually focus on ophiostomatalean mutualists (as discussed in Davis 2015; Saucedo-Carabez et al. 2018). Although symbiotic yeasts are important associates of other beetle taxa (Pant and Fraenkel 1950; Suh et al. 2005; Shukla et al. 2018), the implication of this yeast-beetle interaction for both organisms is still unknown and the association between *Starmera* sp. and *E. parallelus* only contributes to this puzzle.

One female gut yielded *R. xyleborini*, although only at a low CFU count. The ambrosia-symbiotic fungus *Dryadomyces sulphureus* (=*Raffaelea sulphurea*) was reported as occurring in the gut of its vector beetle, *Xyleborinus saxesenii*, in as many as 70% of the beetles sampled (Biedermann et al. 2013). Various other fungi have also been observed in the guts of other Scolytinae and Platypodinae beetles (Kirkendall et al. 2015; Peris et al. 2021). We found no systematic occupation of the gut in *E. parallelus* by potential symbiotic partners, therefore our findings suggest that the gut is not likely to be a reliable mode of transport for the fungal mutualist.

Assigning fungal mutualist(s)-beetle species parings often includes ambiguity, and so it requires replicated sampling (Skelton et al. 2018). This ambiguity seems to be partly due to a certain degree of promiscuity in *Raffaelea*, but also due to inconsistent recovery of symbionts in the laboratory, likely a consequence of the variable symbiont load of different dispersal stages of the beetle (Bateman et al. 2015; Skelton et al. 2018). In our study *E. compositus* and *E. parallelus* were repeatedly and predominantly associated with a single *Raffaelea* species (*R. xyleborini*), with *Dryadomyces*, *Harringtonia* and several *Raffaelea* species also recovered albeit with lesser prevalence. While some members of these genera have been shown to be nutritional mutualists of other ambrosia beetles, others such as the globally distributed *R. subalba* appear to be promiscuous among unrelated beetle vectors and could be commensals or parasites (Hulcr and Stelinski 2017). Like in the Scolytinae ambrosia beetles and their mutualists (Saucedo-Carabez et al. 2018), the presence of one dominant and consistent mutualist and multiple commensal *Ophiostomatales* seems to be an emerging feature of the ambrosial symbiosis within Platypodinae (Li et al. 2018a).

The dominant fungus recovered from both *Euplatypus* species was putatively identified as *R. xyleborini*. This fungus was first isolated from *X. andrewesi* in Florida as an unidentified *Raffaelea* isolate (Bateman et al. 2015) and later described as *R. xyleborini* (Simmons et al. 2016) (originally named *R. xyleborina*, an orthographic variant of the name). Accumulating evidence suggests, however, that this fungus is a primary mutualist of *Euplatypus*, not *Xyleborinus.* This fungal species was found on *Euplatypus* specimens from Belize, China, Florida (Li et al. 2018a; this study), and Brazil (this study). Based on our results, it appears that *R. xyleborini* was only incidental to *X. andrewesi*, which is not native to Florida. We caution against the practice of naming ambrosia fungi after vector beetles before the connection is unambiguously established.

Although most beetle and fungal species display high to intermediate fidelity, horizontal transfer of fungal mutualists (and commensals) also occurs in Scolytinae (Mayers et al. 2022). This is more apparent when cross-contamination by a phytopathogenic fungus occurs between exotic and native beetle species (Carrillo et al. 2014), as it is the case of the causative agent of laurel wilt, *Harringtonia lauricola* (=*Raffaelea lauricola*) (Saucedo-Carabez et al. 2018). In the United States, *H. lauricola* was introduced via the scolytine *Xyleborus glabratus* that was originally from southeastern Asia. However, *H. lauricola* has now been documented and vectored by several species of native scolytines from North America. In our survey we did not find *H. lauricola* in *E. parallelus* or *E. compositus* sampled in Florida. Similarly, *H. lauricola* was absent from the mycangia of other North American platypodine that have been surveyed (Ángel-Restrepo et al. 2022), despite the fact that *E. parallelus* visits the same plant hosts shared by other Scolytinae beetles that act as vectors of *H. lauricola*. This suggests that even though some fungi seem to be promiscuous and are sometimes found within multiple beetle species, their promiscuity may still be limited to specific beetle clades.

A species of *Ceratocystiopsis* was also found in moderate abundance in the head and prothorax of *E. compositus*

and *E. parallelus*. In our phylogenetic analysis, the isolates clustered with isolates named either *C. lunata* or *C. quercina. Ceratocystiopsis quercina* was described based on the sexual morph (Inácio et al. 2022), and shares 100% identity with *C. lunata* in both LSU and ß-tubulin gene sequences. Based on the morphological description by Inácio et al. (2022) and the fact that these authors did not include *C. lunata* in their phylogenetic analysis, it seems likely that *C. quercina* is a later synonym of *C. lunata*. In addition, our phylogenetic analysis grouped *C. lunata* and *C. quercina* into a well-resolved clade, sister to other *Ceratocystiopsis* species (Online Resource Fig. S3). This is similar to the results from other recent studies (Li et al. 2018a; Nel et al. 2021). Interestingly, we recovered *C. lunata* on culture medium amended with cycloheximide (Harrington et al. 2010), demonstrating that it is resistant to this antifungal compound, even though most *Ceratocystiopsis* species are sensitive to cycloheximide (Hausner et al. 1993; Zipfel et al. 2006). Additional taxonomic studies are necessary to elucidate the diversity of species in this genus and their phylogenetic relationships. Species in *Ceratocystiopsis* have been consistently found as opportunistic commensals of bark beetles, even obligate mutualists in some cases (Zipfel et al. 2006; Yamaoka et al. 2009; Six 2012; Six and Elser 2020). Further expanding the distribution of the genus, *C. lunata* was isolated from the ambrosia beetles *X. crassiusculus* in South Africa (Nel et al. 2021) and *Monarthrum conversum* in avocado in Mexico (Ángel-Restrepo et al. 2022). In our phylogenetic analysis, we found that the LSU sequences obtained by Li et al. (Li et al. 2018a) from the platypodinae beetles *Oxoplatypus quadridentatus*, *E. compositus*, and *E. parallelus* in the US were resolved as *C. lunata*. Our findings along with these reports support the hypothesis that *Ceratocystiopsis* species regularly associate with species of Platypodinae.

5 Conclusions

Despite an exhaustive search with three complementary techniques, no mycangium was found in *E. parallelus*. This seems surprising given that *E. parallelus* is associated with a consistent community of symbionts. Notably, a novel association between *E. compositus* and *E. parallelus* with a *Starmera* yeast in the hindgut of dispersing females suggests a previously undocumented, and possibly nutritional, relationship. This study adds to the growing evidence that Platypodinae beetles harbor an extraordinary diversity of fungi and fungus-insect symbiotic relationships, most of which remain unstudied.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-023-00938-4.

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Authors' contributions AR, AJJ, JH designed the study. JH, MES, and NOK funding and contributed resources for this work. AR, AJJ, RAJ, and YL carried out fieldwork. AR, BW, ELS, MK, MW, and RAJ carried out laboratory work. AR and AJJ organized and analyzed the data. AR, AJJ and JH wrote the first drafts of the manuscript. All authors revised and contributed to writing process of the manuscript.

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Data Availability The datasets supporting the results in the paper are available in the Supplementary Material (Tables S1 and S2). DNA sequences generated in this study are deposited in NCBI-GenBank (accessions available in Table S2).

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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