New Raffaelea species (Ophiostomatales) from the USA and Taiwan associated with ambrosia beetles and plant hosts

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Abstract: Raffaelea (Ophiostomatales) is a genus of more than 20 ophiostomatoid fungi commonly occurring in symbioses with wood-boring ambrosia beetles. We examined ambrosia beetles and plant hosts in the USA and Taiwan for the presence of these mycosymbionts and found 22 isolates representing known and undescribed lineages in Raffaelea. From 28S rDNA and β-tubulin sequences, we generated a molecular phylogeny of Ophiostomatales and observed morphological features of seven cultures representing undescribed lineages in Raffaelea s. lat. From these analyses, we describe five new species in Raffaelea s. lat.: R. aquacuta, R. campbellii, R. crostotarsa, R. cyclorhipidia, and R. xyleborina spp. nov. Our analyses also identified two plant-pathogenic species of Raffaelea associated with previously undescribed beetle hosts: (1) R. quercivora, the causative agent of Japanese oak wilt, from Cyclorrhizidion ohnoi and Crossotarsus emancipatus in Taiwan, and (2) R. lauricola, the pathogen responsible for laurel wilt, from Ambrosiodmus lecontei in Florida. The results of this study show that Raffaelea and associated ophiostomatoid fungi have been poorly sampled and that future investigations on ambrosia beetle mycosymbionts should reveal a substantially increased diversity.

Key words: entomogenous fungi, insect-fungus interactions, Japanese oak wilt, laurel wilt, molecular phylogenetics, mycosymbioses

INTRODUCTION

Raffaelea (Arx & Hennebert 1965) is a genus of primarily asexual fungi including more than 20 species in Ophiostomatales (Harrington et al. 2010, de Beer et al. 2013, Musvuugwa et al. 2015). These fungi commonly occur in symbioses with wood-boring ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae). Ambrosia beetles propagate these and other fungi, which obtain nutrients from plant tissues and provide the beetles with a food source, throughout galleries in their plant hosts. When female beetles leave the parental gallery to establish a new generation, they transport inocula of one or several mycosymbionts in specialized cavities in various parts of their bodies, to be grown in the subsequently developed galleries (Hubbard 1897, Beaver 1989).

The asexual morphological characteristics of Raffaelea are rather simple: hyaline, rarely-branching, commonly single-celled conidiophores are arranged singly or aggregated in sporodochia; conidiogenous cells are precociously or sympodially proliferating, which may leave dendrites, annelations, or inconspicuous scarring; conidia range from elliptical to globose, with some exceptions, and may reproduce by yeast-like budding (Harrington et al. 2010, Musvuugwa et al. 2015). De Beer & Wingfield (2013) recognized two sexually reproducing species of Ophiostoma, O. seticollis and O. deltoideosporum, in Raffaelea s. str. based on DNA sequence phylogenies, but they did not transfer these species to Raffaelea. Subsequently, Musvuugwa et al. (2015) described a Raffaelea species, R. vaginata, with an observed sexual morph, similar to those of O. seticollis and O. deltoideosporum. The latter authors consequently emended the circumscription of the genus to include both asexual and sexual morphs, and transferred the two Ophiostoma species to Raffaelea as R. seticollis and R. deltoideosporum, consistent
Table 1. Cultures of *Raffaelea* examined in our molecular phylogenetic analyses, with ex-type cultures of new species in bold.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>CMW culture number (Other identifier)</th>
<th>Beetle host</th>
<th>Plant host</th>
<th>Country</th>
<th>Region (Locality)</th>
<th>28S rDNA</th>
<th>BTUB</th>
<th>18S rDNA</th>
<th>ITS DNA</th>
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<td><em>R. aguacate</em></td>
<td>PL1004</td>
<td>CMW38067 (272)</td>
<td></td>
<td></td>
<td></td>
<td>Florida (Miami-Dade Co.)</td>
<td>KJ909296</td>
<td>KJ909297</td>
<td>KF026302</td>
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<tr>
<td><em>R. cf. campbellii</em></td>
<td>Hulcr7355</td>
<td>Euplatypus compositus</td>
<td>USA</td>
<td>Florida (Miami-Dade Co., Homestead)</td>
<td></td>
<td>KX267101</td>
<td>KX267112</td>
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<td><em>R. cf. subalba</em></td>
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<td>Euplatypus compositus</td>
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<td><em>R. crossotarsa</em></td>
<td>Hulcr7182</td>
<td>CMW44793</td>
<td>Crototarsus emancipatus</td>
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<td>Fushan</td>
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<td>KX267114</td>
<td>KX267129</td>
<td>KX267135</td>
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<td><em>R. cyclorhipidia</em></td>
<td>Hulcr7168</td>
<td>CMW44790</td>
<td>Cyclorhipidion ohnol</td>
<td>Lithocarpus sp.</td>
<td>Taiwan</td>
<td>Fushan</td>
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<td>CMW44791</td>
<td>Ambrosiodmus lecontei</td>
<td>Persea borbonia</td>
<td>USA</td>
<td>Florida (Lake Kissimmee)</td>
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<td>Xyleborinus glabratus</td>
<td>Taiwan</td>
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<td>KX267117</td>
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<td>Xyleborinus glabratus</td>
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<td>Cyclorhipidion ohnol</td>
<td>Lithocarpus sp.</td>
<td>Taiwan</td>
<td></td>
<td>Fushan</td>
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<td>KX267119</td>
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<td>Cyclorhipidion ohnol</td>
<td>Lithocarpus sp.</td>
<td>Taiwan</td>
<td></td>
<td>Fushan</td>
<td>KX267108</td>
<td>KX267120</td>
<td>KX267132</td>
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<tr>
<td><em>R. subfuscra</em></td>
<td>Hulcr4520</td>
<td>(C2335)</td>
<td>Xyleborinus glabratus</td>
<td>USA</td>
<td>South Carolina (Hunting Island State Park)</td>
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<td>KX267137</td>
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<td>Hulcr4717</td>
<td>Euplatypus compositus</td>
<td>USA</td>
<td>Virginia (Albemarle Co., Batesville)</td>
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<td>KX267133</td>
<td>KX267138</td>
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<td>Hulcr4719</td>
<td>Euplatypus compositus</td>
<td>USA</td>
<td>Pennsylvania (Huntingdon Co., Raystown Lake)</td>
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<td>KX267123</td>
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<td><em>R. xyleborina</em></td>
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<td>CMW445859</td>
<td>Xyleborinus andrewesi</td>
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<td>Florida (Highlands Co., Venus)</td>
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<td>KX267124</td>
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<td>Hulcr6100</td>
<td>Xyleborinus andrewesi</td>
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<td>Florida (Highlands Co., Venus)</td>
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<td>Hulcr6406</td>
<td>Xyleborinus andrewesi</td>
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<td><em>Raffaelea sp.</em></td>
<td>Hulcr5951</td>
<td>(PL1635)</td>
<td>Xyleborinus pinicola</td>
<td>Pinus keyseri</td>
<td>Thailand</td>
<td>Mae Chaem</td>
<td>KJ909308</td>
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<td><em>Raffaelea sp.</em></td>
<td>Hulcr7507</td>
<td>Xyleborinus gracilis</td>
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<td>Florida</td>
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<td><em>Raffaelea sp.</em></td>
<td>PL1001</td>
<td>CMW38062</td>
<td>Persea americana</td>
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</table>
with the one fungus-one name rule (Hawksworth 2011).

Some molecular phylogenies of Raffaelea and additional genera within Ophiostomatales have suggested that Raffaelea is monophyletic (e.g. Harrington et al. 2010). However, more recent and comprehensive analyses (de Beer & Wingfield 2013, Dreaden et al. 2014, Musvuugwa et al. 2015) have shown that Raffaelea species constitute three clades in the order, Raffaelea s. str., the R. lauricola complex, and the R. sulphurea complex. Of ecological interest, the two clades exterior to Raffaelea s. str. each include plant pathogens that have been spread in the last decade by their respective insect vectors. Raffaelea lauricola, the causative agent of laurel wilt in the southeastern USA, is associated with the ambrosia beetle Xyleborus glabratus (Harrington et al. 2008, Ploetz et al. 2013), among others (Carrillo et al. 2014). Raffaelea lauricola, the eponymous member of the R. lauricola complex (de Beer & Wingfield 2013, Musvuugwa et al. 2015), is sometimes placed as sister to Raffaelea s. str. in molecular phylogenies of individual rDNA loci (Musvuugwa et al. 2015) and additional coding genes (Dreaden et al. 2014). Raffaelea quercivora, which is responsible for Japanese oak wilt and associated with Platypus quercivorus (Kubono & Ito 2002, Kusumoto et al. 2014), lies within the R. sulphurea complex in Leptographium s. lat. (de Beer & Wingfield 2013, Dreaden et al. 2014, Musvuugwa et al. 2015).

During domestic (Campbell et al. 2016) and international studies to investigate the diversity of ambrosia beetles and their fungal symbionts, raffaelea-like isolates from the southeastern USA and Taiwan were collected; preliminary molecular analyses indicated that some of these isolates represent novel lineages within Raffaelea s. lat. In this study, we use nine isolates to describe five new species in Raffaelea from collections of plant hosts and ambrosia beetles. We have also characterized 13 additional Raffaelea isolates based on DNA sequence data.

MATERIALS AND METHODS

DNA extraction, PCR amplification and sequencing

Twenty-two Raffaelea cultures and DNA extracts were aggregated from the Forest Entomology laboratory at the University of Florida (Gainesville, FL) and the University of Florida’s Tropical Research and Education Center (Homestead, FL). Cultures from ambrosia beetle hosts were isolated by dilution plating of mycangial contents, as described by Li et al. (2015). Cultures of newly described species are deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Fungal DNA was isolated with Extract-N-Amp PCR kits (Sigma-Aldrich), as described by Li et al. (2015). Final concentrations of PCR reagent solutions in 25 µL were: (1) 1× ClonTech-TaqKara Ex Taq Buffer; (2) 5 % DMSO; (3) 0.2 mM each dNTP; (4) 0.5 µM each primer; (5) 0.625 U Ex Taq polymerase; and (6) 0.01–0.1 ng extracted DNA. Primer combinations used for amplifications were: (1) LROR/LR5 (Vigaly & Hester 1990, Rehner & Samuels 1994) for nuclear large subunit (28S) ribosomal DNA (rDNA); (2) T10 or Bt2a/Bt2b (Glass & Donaldson 1995, O’Donnell & Cigelnik 1997) for β-tubulin (βT); (3) NS1/NS4 for nuclear small subunit (18S) rDNA; and (4) either ITS3/LR3 or ITS1F/ITS4 (White et al. 1990, Gardes & Bruns 1993) for portions of the ITS1-5.8S-ITS2 (ITS) rDNA locus. The PCR conditions for βT and ITS rDNA were the same as those used by Yin et al. (2015) and for 18S and 28S rDNA by Dreaden et al. (2014). Amplified products were visualized and purified as described by Li et al. (2015), and these were submitted to the University of Florida Interdisciplinary Center for Biotechnology Research for Sanger sequencing. Chromatograms were assembled and inspected with Geneious v. 9.0.5.

Phylogenetic analyses

Sequences of 28S rDNA and βT (introns 3/4/5 removed) were aligned and visually inspected in Geneious for phylogenetic reconstruction. The alignment was divided into four partitions for phylogenetic consideration: one partition for the 28S rDNA alignment and for each of the three codon positions in the protein encoding βT. The Akaike information criterion in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) was used to select the nucleotide substitution model for each partition. Maximum likelihood (ML) phylogenetic analyses were conducted in GARLI 2.01 (Zwickl 2006) with the recommended partition parameters to determine the best tree topology (Fig. 1) and bootstrap support values from 500 search replicates, which were summarized in SumTrees (Sukumaran & Holder 2010). Bayesian posterior probabilities (BPP) were estimated with the same partition parameters in an analysis conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), in which two runs of four chains each were executed simultaneously for 5 000 000 generations, with sampling every 500 generations. SumTrees was used to compute BPP from a summary of 7501 trees retained after a burn-in of the first 2500 trees collected.

Growth trials and morphological characterization

To determine optimal growth rates of each new species of Raffaelea, discs of agar (7 mm diam) covered with mycelium were aseptically removed from 1-wk-old cultures growing on BD Difco™ MEA and used to inoculate plates incubated at 10–35 °C, in 5 °C intervals. After 9 d, colony growth was calculated as by Musvuugwa et al. (2015). Cultures of newly described species were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Morphological features were examined by inoculating sterile slide mounts of BD Difco™ MEA with propagules collected by running a sterile needle along the surface of cultures growing on BD Difco™ MEA. Once reproductive structures were observed, discs of agar (7 mm diam) covered with mycelium were aseptically removed from 1-wk-old cultures growing on BD Difco™ MEA and used to inoculate plates incubated at 10–35 °C, in 5 °C intervals. After 9 d, colony growth was calculated as by Musvuugwa et al. (2015). Growth trials and morphological characterization

RESULTS

All isolates we examined resided in Raffaelea s. str., the R. lauricola complex, or the R. sulphurea complex in the
Fig. 1. Best ML tree from GARLI analysis of 28S rDNA and βT data matrix of Ophiostomatales genera with Sordariales as outgroup (Musvuugwa et al. 2015). Values at nodes represent ML bootstrap percentages ≥ 70 % from a summary of 500 replicates, and branches in bold represent BPP ≥ 95 %. L denotes GenBank accession number of 28S rDNA sequence for taxon; B denotes GenBank accession number of βT sequence for taxon. Types of genera and new species of Raffaelea in bold.

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phylogenetic analyses of the 28S rDNA and βT data matrices (Fig. 1). The \textit{R. lauricola} complex was sister to the \textit{Raffaelea s. str.} clade with 81 % ML bootstrap and 100 % BPP support, and the well-supported \textit{R. sulphurea} clade resolved within \textit{Leptographium s. lat.} The ITS and 18S rDNA sequences were not included in the phylogenetic analyses, but these sequences were used for molecular identification (Table 1). The data matrix for the 28S rDNA and βT regions has been deposited in TreeBASE as submission 19323.

The new species in \textit{Raffaelea s. str.} and the \textit{R. lauricola} complex (Table 1) possessed all βT introns (3/4/5). Isolates Hulcr7167 and Hulcr7176 possessed two introns (3/4/-). These patterns of intron presence were the expected conditions for the majority of species in each clade (de Beer & Wingfield 2013). Although they were not isolated from \textit{Platypus quercivorus}, isolates Hulcr7167 and Hulcr7176 resolved in the \textit{R. sulphurea} complex with \textit{R. quercivora}, and were 99 % (396/400 bp) and 98 % (392/400 bp) similar, respectively, to the \textit{R. quercivora} sequence (GenBank accession AB496454) had six ambiguous bases that increased the level of dissimilarity with our isolates.

\section*{TAXONOMY}

\textbf{\textit{Raffaelea aguacate} D.R. Simmons, Dreaden & Ploetz, sp. nov.}

\textit{MycoBank MB817170} (Fig. 2)

\textit{Etymology:} The epithet “aguacate” refers to the Spanish for avocado (\textit{Persea americana}), from which this isolate was cultured.

\textit{Diagnosis:} Conidiogenous cells 13 (±2) × 2.7 (±0.3) µm, hyaline, sometimes irregular; conidia at conidiogenous cell apex or sessile and lateral; conidia 7.2 (±0.6) × 2.6 (±0.5) µm, elongate, truncated at base, hyaline, rarely with yeast-like budding.

\textit{Type: USA: Florida: Miami-Dade Co., Homestead, from bioassay of \textit{Persea americana}, 2009, C. L. Harmon (BPI 910154 – holotype; 272 = PL1004 = CMW38067 – ex-type cultures).}

\textit{Description:} Colonies initially cream, turning light green to olivaceous, aging to dark green on MEA; reverse subhyaline. Optimal colony diameter after 9 d at 25 °C in the dark was 70.2 (±3.9) mm; 46.0 (±2.6) mm at 10 °C; no growth at 35 °C.

\textit{Conidiogenous cells} hyaline, sometimes irregular, tapering at ends, 13 (±2) × 2.7 (±0.3) µm. \textit{Conidia} forming from apex of conidiogenous cells, hyaline, occasionally sessile and lateral. Conidia produced singly, aseptate, elongate, and occasionally truncated at the base, 7.2 (±0.6) × 2.6 (±0.5) µm. Conidia rarely budding. \textit{Sexual morph} unknown.

\textbf{\textit{Raffaelea campbellii} D.R. Simmons, A. Campbell & Ploetz, sp. nov.}

\textit{MycoBank MB817171} (Fig. 3)

\textit{Etymology:} The epithet “campbellii” is in honor of Donald and Princesa Campbell, parents of Alina S. Campbell, collector of the specimen, for their guidance and support.

\textit{Diagnosis:} Conidiogenous cells 13.7 (±1.6) × 3.7 (±0.3) µm, hyaline, flask-shaped; conidia at conidiogenous cell apex; conidia 6.7 (±1.2) × 3.6 (±0.5) µm, ovoid to elliptical, truncated at base, hyaline.

\textit{Type: USA: Florida: Miami-Dade Co., cultured from \textit{Xyleborus glabratu} that infected \textit{Persea palustris}, Jun. 2013, A. S. Campbell (BPI 910156 – holotype; 103p2 = CMW44800 – ex-type culture).}

\textit{Additional specimen examined:} Loc. cit (110p2 = CMW44801).

\textit{Description:} Colonies initially cream, turning olivaceous to blackish on MEA, surface tough and wrinkled; reverse subhyaline. Optimal colony diameter after 9 d at 25 °C in the dark 25.7 (±3.9) mm; no growth at 10 °C or 35 °C.
Conidiogenous cells hyaline, flask-shaped, tapering towards the apex, 13.7 (±1.6) × 3.7 (±0.3) µm. Conidia forming from apex of conidiogenous cells, hyaline. Conidia produced singly, accumulating at tip of conidiogenous cells, aseptate, ovoid to elliptical, sometimes fusiform, and often truncate at the base, 6.7 (±1.2) × 3.6 (±0.5) µm. Sexual morph unknown.

**Raffaelea crossotarsa** D.R. Simmons & Y.T. Huang, sp. nov. MycoBank MB817172 (Fig. 4)

*Etymology:* The epithet “crossotarsa” refers to the genus of the host beetle (*Crossotarsus emancipatus*), the mycangium of which yielded this fungus.

*Diagnosis:* Conidiogenous cells 15.2 (±2.1) × 3 (±0.3) µm, hyaline, slender; conidium at conidiogenous cell apex; conidia 6 (±0.4) × 4.9 (±0.3) µm, globose to ovoid, hyaline, yeast-like budding from prominent isthmus.

*Type:* Taiwan: Fushan, cultured from *Crossotarsus emancipatus* collected from *Lithocarpus* sp., Mar. 2015, J. Hulcr, A. Black & D. R. Simmons (BPI 910157 – holotype; Hulcr7182 = CMW44793 – ex-type culture).

**Description:** Colonies initially cream, aging from golden olivaceous to dark green or dark ruddy brown on MEA, surface tough; reverse subhyaline. Optimal colony diameter after 9 d at 25 °C in the dark was 39.2 (±1.2) mm; 9.0 (±0.5) mm at 10 °C; no growth at 35 °C. **Conidiogenous cells** hyaline, slender, tapering at ends, 15.2 (±2.1) × 3 (±0.3) µm. **Conidia** forming from apex of conidiogenous cells, hyaline. Conidia produced singly, aseptate, globose to ovoid, 6 (±0.4) × 4.9 (±0.3) µm. Conidia producing budding cells from prominent isthmuses, 1–2 µm long. Sexual morph unknown.

**Raffaelea cyclorhipidia** D.R. Simmons & Y.T. Huang, sp. nov. MycoBank MB817173 (Fig. 5)

*Etymology:* The epithet “cyclorhipidia” refers to the genus of the host beetle (*Cyclorhipidion ohnoi*), the mycangium of which yielded this fungus.

*Diagnosis:* Conidiogenous cells 12 (±1.7) × 3.6 (±0.3) µm, hyaline, flask-shaped; conidium at conidiogenous cell apex or sessile and lateral; conidia 7.3 (±1.0) × 3.5 (±0.7) µm, elliptical to elongate, hyaline, yeast-like budding.

*Type:* Taiwan: Fushan, cultured from *Cyclorhipidion ohnoi*
**Raffaelea**

spp. nov. from the USA and Taiwan

**ARTICLE**

**Description:** Colonies initially cream, aging from olivaceous to golden brown or blackish on MEA, surface tough and wrinkled; reverse subhyaline. Optimal colony diameter after 9 d at 25 °C in the dark was 47.5 (±1.9) mm; 20.6 (±1.8) mm at 10 °C; no growth at 35 °C. Conidiogenous cells hyaline, flask-shaped, tapering towards the apex, 12 (±1.7) × 3.6 (±0.3) µm. Conidia forming at apex of conidiogenous cells, occasionally sessile and lateral, hyaline. Conidia produced singly, aseptate, elliptical to elongate, occasionally truncate at base, 7.3 (±1.0) × 3.5 (±0.7) µm. Conidia produce budding cells. Sexual morph unknown.

**Raffaelea xyleborina** D.R. Simmons & C. Bateman, sp. nov. MycoBank MB817174 (Fig. 6)

**Etymology:** The epithet “xyleborina” refers to the genus of the host beetle (*Xyleborinus andrewesii*), the mycangium of which yielded this fungus.

**Diagnosis:** Conidiophores micronematous, hyaline; conidia at conidiogenous cell apex or lateral and sessile; conidia 6.5 (±0.7) × 4.9 (±0.8) µm, globose to ovoid, truncated at base, hyaline, yeast-like budding.

**Type:** USA: Florida: Highlands Co., Venus, cultured from *Xyleborinus andrewesii* collected from bait trap, 3 Jan. 2013, C. Bateman, C. Gibbard & L. L. Stelinski (BPI 910159 – holotype; Hulcr6099 = CMW45859 – ex-type culture; Additional specimens examined: Loc. cit (Hulcr6100, Hulcr6406, Hulcr6408).

**Description:** Colonies initially cream, varying with age from cream to dark green to blackish on MEA, surface tough and spiral in appearance; reverse subhyaline. Optimal colony diameter after 9 d at 35 °C in the dark was 26.8 (±3.0) mm; 14.9 (±1.3) mm at 25 °C; no growth at 10 °C. Conidiogenous cells hyaline, micronematous, with conidia forming at apex, occasionally sessile and lateral. Conidia produced singly, aseptate, hyaline, globose to ovoid, sometimes elongate, and often truncated at base, 6.5 (±0.7) × 4.9 (±0.8) µm. Conidia produce budding cells. Sexual morph unknown.

![Fig. 5. Raffaelea cyclorhipidia (Hulcr7168) morphological features in pure culture on MEA. A. Colony growth after 9 d at 25 °C. B–C. Hyphae bearing typical flask-shaped conidiogenous cells with conidia at apex, and occasional lateral sessile conidia (black arrowheads). D. Elliptical to elongate conidia budding yeast-like daughter cells (black arrows). Bar in B applies also to C–D.](image)

![Fig. 6. Raffaelea xyleborina (Hulcr6099) morphological features in pure culture on MEA. A. Colony growth after 9 d at 25 °C. B. Micronematous conidiogenous cells with ovoid conidia truncated at base (white arrowhead). C. Micronematous conidiophore with short conidiogenous cell sessile at side (black arrowhead) and at apex. D. Globose to ovoid conidia truncated at the base (white arrowhead) and budding yeast-like daughter cells (black arrows). Bar in C applies also to B–D.](image)
DISCUSSION

Considering the damage that ambrosia fungi and their vectors cause (Ploetz et al. 2013), there is an urgent need to determine not only the diversity of these fungi globally but also to gain an enhanced knowledge of the host vector range for these potentially devastating species. Comparison of fungal isolates in this study with known species of Raffaelea revealed that two isolates from Taiwan, Hulcr7167 and Hulcr7176, grouped with R. quercivora. Raffaelea quercivora has been isolated from Platypus quercivorus in Japan, where it is responsible for ongoing epidemics of Japanese oak wilt (Kubono & Ito 2002), as well as in Taiwan (Kusumoto et al. 2014). Our isolates of R. quercivora were not isolated from the mycangia or fungal galleries of P. quercivorus, however, but rather from the mycangia of Cyclorhipidion ohnoi and the fungal galleries of Crossotarsus emancipatus from Taiwan. Though the latter two beetle species have not been implicated in oak wilt, these symbioses suggest that other vectors of R. quercivora exist. These isolates were collected from the same beetle host populations in Taiwan from which two of the species newly described in this study, R. cyclorhipidia and R. crossotarsa, were recovered. Therefore, these beetle-associated species display a degree of promiscuity with fungi within and exterior to Raffaelea s. str.

Raffaelea lauricola, the causative agent of laurel wilt, was found in Taiwan, from the documented host Xyleborus glabratus, and in Florida, from the previously unrecorded host Ambrosiodmus lecontei. Carrillo et al. (2014) reported that R. lauricola was laterally transferred to additional ambrosia beetle hosts, other than X. glabratus, when these species co-inhabit trees infected with this fungal pathogen. This finding demonstrates that the pathogen is a relatively promiscuous symbiont of ambrosia beetles, raising its importance from the biosecurity perspective. Despite Carrillo et al. (2014) having examined 41 adult A. lecontei females emerging from laurel wilt-affected swamp bay bolts, they failed to isolate R. lauricola from this host species. However, we recovered R. lauricola from A. lecontei infesting Persea borbonia near Lake Kissimmee (FL). The presence of Raffaelea with Ambrosiodmus may be phoretic or facultative, because Ambrosiodmus species examined to date carry a highly specific ambrosial basidiomycetous species (Li et al. 2015).

Besides information on known ambrosia fungi, results of this study suggest that under-explored regions of the world contain a large diversity of undescribed ambrosia fungi. Phylogenetic analyses of DNA sequence data for 22 isolates of Raffaelea-like fungi led to the discovery of the five new species described here. Some additional isolates resolved in lineages that would generally support their description as novel taxa (i.e. Hulcr9591; Hulcr7355; Hulcr7507 and PL1001), but these cultures could not be revived for morphological characterization after cryopreservation. Four of the new species described in this study were isolated from mycangia of ambrosia beetle hosts. Although sampling efforts that provided the foundation for this study included many different parts of the world, three of the novel taxa were from the eastern US. Whether this is a true reflection of an unexamined area of Raffaelea species diversity, or due to sampling bias, is unknown but deserves future consideration.

Results from this study indicate that Raffaelea s. str. and the R. lauricola complex are monophyletic (Fig. 1; Raffaelea s. lat.). This is consistent with previous analyses using rDNA and 18S sequences (Dreaden et al. 2014). Analyses of 28S rDNA across Ophiostomatales have shown the same association with some support (Musvuugwa et al. 2015) or that these clades are disparate (de Beer & Wingfield 2013). Until a more accurate determination of their relationship is conducted with additional genetic loci, we conclude that these two clades are distinct.

Fungal symbioses with ambrosia beetles have become especially fertile topics for research, and further study will likely identify an increasingly large diversity of fungal associates. Indeed, Bateman et al. (2016) described a new genus in Ophiostomatales from Premnobiis caviennis (Scolytinae: Ipini), an independently evolved ambrosia beetle lineage largely confined to Africa. Furthermore, ambrosia beetles’ mycosymbionts are not limited to the ascomycetous Ophiostomatales. Li et al. (2015) found a new basidiomycetous Polyporales fungus, Flavodon ambrosius (Simmons et al. 2016), in symbiosis with Ambrosiodmus species, and Kasson et al. (2016) found the same mycosymbiont associated with another genus, Ambrosiophilus, which is sister to Ambrosiodmus (Hulcr & Cognato 2010). Thus, as investigations into these insects increase in number, additional fungal genera in unexpected lineages may be found in symbioses with ambrosia beetles.

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