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# New combinations in *Raffaelea*, *Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*

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**Abstract** — Female adults of the redbay ambrosia beetle, *Xyleborus glabratus* (*Coleoptera: Curculionidae: Scolytinae*), from the southeastern USA were individually macerated and serially diluted onto culture media for isolation of fungal symbionts. Six *Raffaelea* species were recovered: *R. lauricola*, *R. arxii*, and four new species: *R. subalba*, *R. ellipticospora*, *R. fusca* and *R. subfusca*. Phylogenetic analyses of LSU rDNA sequences placed these mycangial inhabitants and other species of *Raffaelea*, as well as some species of *Ambrosiella* associated with ambrosia beetles, into a monophyletic, asexual clade within *Ophiostoma*. New combinations in *Raffaelea* are made for some *Ambrosiella* species and *Dryadomyces amasae*. Ambrosia beetle symbionts with *Ceratocystis* affinities, including *A. trypodendri* comb. nov., are retained in *Ambrosiella*, but *Ambrosiella* species associated with bark beetles are transferred to the anamorph genus *Hyalorhinocladiella* as *H. ips, H. macrospora*, and *H. tingens*.

**Key words** — *Grosmannia, Leptographium, Ophiostomataceae, Ophiostomatales*, *Scolytidae*

## Introduction

The ecology of only a small fraction of the approximately 3400 species of ambrosia beetles (*Coleoptera: Curculionidae: Scolytinae* and *Platypodinae*) has been studied in detail (Batra 1963, Farrell et al. 2001, Francke-Grosmann 1967), and relatively few of their fungal symbionts have been described (Batra 1968, Massoumi Alamouti et al. 2009). Ambrosia beetles are polyphyletic and were derived from bark beetles in at least seven evolutionary events (Farrell et al. 2001). Ecologically, ambrosia beetles are distinguished from bark beetles by laying eggs along tunnels in the sapwood of dead or dying trees, while bark

beetles lay their eggs along galleries in the nutrient-rich inner bark (phloem) of trees (Harrington 2005). Ambrosia beetle adults and larvae feed on symbiotic fungi that grow in the otherwise nutrient-poor sapwood (Batra 1963, Francke-Grosmann 1967). The symbionts produce small conidiophores in tight clusters (sporodochia), which are suitable for grazing by ambrosia beetle larvae and adults (Batra 1968, Harrington 2005). Budding spores of the fungal symbionts are carried in one or both sexes of adult ambrosia beetles in specialized sacs called mycangia (Batra 1963, Beaver et al. 1989, Francke-Grosmann 1967, Six 2003). The fungal symbionts of the beetles are asexual (Batra 1963), and their reduced morphology has led to ambiguous classification systems, at least until the common application of DNA sequence analyses (Cassar & Blackwell 1996, Jones & Blackwell 1998, Rollins et al. 2001).

A comprehensive taxonomic evaluation of fungi associated with ambrosia beetles has not been conducted since Batra (1968), who placed most of the known species in the anamorph genera *Ambrosiella* and *Raffaelea*. The type species of these genera are placed by phylogenetic analyses within the ascomycete genera *Ceratocystis* Ellis & Halst. and *Ophiostoma* Syd. & P. Syd., respectively (Cassar & Blackwell 1996, Jones & Blackwell 1998). Most of the ambrosia beetle symbionts fall within the *Ophiostoma* clade (Gebhardt et al. 2005, Kolarik & Hulcr 2009, Massoumi Alamouti et al. 2009). Traditionally, species of *Ambrosiella* and *Raffaelea* have been distinguished from other anamorphs of *Ophiostoma* based on the clustering of conidiophores into sporodochia, an adaptation for serving as food for insect grazers (Harrington 2005). However, sporodochium formation is found in at least three lineages within the *Ophiostoma* group, and sporodochial anamorphs of *Ophiostoma*-like species could be better split by their ambrosia beetle vs. bark beetle associations (Harrington 2005, Harrington et al. 2008, Massoumi Alamouti et al. 2009).

*Ambrosiella* and *Raffaelea* were originally distinguished based on annellidic vs. sympodial proliferation of the conidiogenous cells, respectively (Batra 1968). However, many *Raffaelea* species have percurrent (annellidic) proliferation of conidiogenous cells (Gebhardt & Oberwinkler 2005), and Batra's distinction appears to have little taxonomic value (Harrington 2005, Harrington et al. 2008). The type species of *Ambrosiella* (*A. xylebori*) is within the *Ceratocystis* clade, and true *Ambrosiella* species produce conidia from deep-seated phialides (Gebhardt et al. 2005, Harrington 2009, Kolarik & Hulcr 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009). Most of the ambrosia beetle symbionts related to *Ophiostoma* species, including the type species of *Raffaelea*, have been described as species of *Raffaelea* (Kubono & Ito 2002, Massoumi Alamouti et al. 2009). Species of *Raffaelea*, along with some *Ambrosiella* species and *Dryadomyces amasae*, appear to form a monophyletic group within *Ophiostoma* (Gebhardt et al. 2005, Massoumi Alamouti et al.

2009). Harrington et al. (2008) emended *Raffaelea* to include all ambrosia beetle symbionts related to *Ophiostoma*.

It is generally believed that only one or a few fungal symbionts are tightly associated with a particular ambrosia beetle species (Batra 1963, Funk 1970). However, our isolations (Harrington & Fraedrich, unpublished) from adult *Xyleborus glabratus* Eichh., the redbay ambrosia beetle, resulted in six species of *Raffaelea*. The most commonly isolated species was *R. lauricola*, which causes laurel wilt on redbay [*Persea borbonia* (L.) Spreng.] and other species in the *Lauraceae* in the southeastern USA (Fraedrich et al. 2008, Harrington et al. 2008). Thus far, *R. lauricola* is the only true vascular wilt fungus associated with an ambrosia beetle (Fraedrich et al. 2008). The beetle is native to Asia (e.g., India, Japan, and Taiwan), usually associated with aromatic plant species, especially species in the family *Lauraceae* (Wood & Bright 1992). The redbay ambrosia beetle was first discovered near Savannah, Georgia, USA, probably introduced in solid wood packing material. Adult females have paired, mandibular mycangia (Fraedrich et al. 2008), and *R. lauricola* can be readily recovered and quantified from beetles by grinding the head of the beetles and dilution plating. Like other ambrosia beetle symbionts, *R. lauricola* can grow in a yeast phase within the mycangium of its ambrosia beetle (Fraedrich et al. 2008, Harrington 2005).

Here we describe four new species of *Raffaelea* isolated from *X. glabratus* recovered from redbay in South Carolina, Georgia, and Florida. Analyses of rDNA sequences infer that these four new species are members of a monophyletic group of ambrosia beetle symbionts that are asexual species of *Ophiostoma*. All beetle symbionts described in the genera *Raffaelea* and *Ambrosiella* are reevaluated taxonomically. Species associated with bark beetles that were previously described as *Ambrosiella* are transferred to *Hyalorhinocladiella*.

## Materials and methods

### Cultures

Adult, female *X. glabratus* were excavated from naturally infested trees of *P. borbonia* with laurel wilt. Beetles were individually macerated in glass tissue grinders, the macerate was serially diluted, and aliquots of the dilutions were plated on malt extract (1% Difco malt extract) agar amended with 200 ppm cycloheximide and 100 ppm streptomycin (CSMA) in 90 mm diameter Petri dishes (Harrington 1992). Cycloheximide media are semi-selective for *Ophiostoma* but do not allow for growth of *Ceratocystis* species or true *Ambrosiella* species (Cassar & Blackwell 1996, Harrington 1981). Representatives of different mycelial phenotypes on CSMA were transferred to separate plates and deposited in the collection of the senior author, and at least three isolates of each putative species were used for rDNA sequencing (Table 1). Cultures of other *Raffaelea* and *Ambrosiella* species were obtained from the Centraalbureau voor Schimmelcultures (CBS) (Table 1).

Table 1. Collection numbers, location, associated insect, SSU and LSU rDNA GenBank accession numbers, and new combinations and synonyms for isolates of *Ambrosiella*, *Raffaelea*, *Ophiostoma*, and *Leptographium*.

| Species | New Combinations and synonyms | Isolate number[A] | Location | Associated insect | SSU Sequence | LSU Sequence |
|---|---|---|---|---|---|---|
| *A. brunnea* | ≡ *Raffaelea brunnea* | C2229, CBS 378.68 | Unknown | *Monarthrum* sp. | EU170280 | EU177457 |
| *A. gnathotrichi* | ≡ *R. gnathotrichi* | C2219, CBS 379.68* | Colorado, USA | *Gnathotrichus retusus* | EU170282 | EU177460 |
| *A. ips* | ≡ *Hyalorhinocladiella ips* | C1572, CBS 435.34* | Minnesota, USA | *Ips* sp. | EU170276 | |
| *A. macrospora* | ≡ *H. macrospora* | C2231, CBS 367.53 | Sweden | *I. acuminatus* | EU170284 | EU177468 |
| *A. sulcati* | = *R. canadensis* | C592, CBS 805.70* | British Columbia, Canada | *G. sulcatus* | EU170281 | EU177459 |
| *A. sulphurea* | ≡ *R. sulphurea* | C593, CBS 380.68* | Kansas, USA | *Xyleborus saxeseni* | EU170272 | EU177463 |
| *A. tingens* | ≡ *H. tingens* | C2232, CBS 366.53 | Sweden | Insect tunnel | EU170277 | EU177474 |
| *R. albimanens* | | C2223, CBS 271.70* | South Africa | *Platypus externedentatus* | EU170269 | EU177452 |
| *R. ambrosiae* | | C2225, CBS 185.64* | United Kingdom | *Platypus cylindrus* | EU170278 | EU177453 |
| *R. arxii* | | C2218, CBS 273.70* | South Africa | *X. torquatus* | EU170279 | EU177454 |
| | | C2372 | Georgia, USA | *X. glabratus* | | EU177455 |
| | | C2398 | South Carolina, USA | *X. glabratus* | | EU177456 |
| *R. canadensis* | | C2233, CBS 168.66* | British Columbia, Canada | *P. wilsonii* | EU170270 | EU177458 |
| | | C2224, CBS 326.70 | South Africa | *P. externedentatus* | EU170275 | EU177467 |
| *R. ellipticospora* | | C2346 | South Carolina, USA | *X. glabratus* | | EU177444 |
| | | C2350 | South Carolina, USA | *X. glabratus* | | EU177445 |
| | | C2395, CBS 121569* | South Carolina, USA | *X. glabratus* | | EU177446 |
| *R. fusca* | | C2254 | Florida, USA | *X. glabratus* | | EU177447 |
| | | C2336 | South Carolina, USA | *X. glabratus* | | EU177448 |
| | | C2394, CBS 121570* | South Carolina, USA | *X. glabratus* | | EU177449 |
| *R. lauricola* | | C2203 | South Carolina, USA | *X. glabratus* | EU123076 | |

| | | | | | |
|---|---|---|---|---|---|
| | C2204 | South Carolina, USA | *X. glabratus* | EU170266 | |
| | C2214 | South Carolina, USA | *X. glabratus* | | |
| | C2227 | Georgia, USA | *Xylosandrus crassiusculus* | EU170267 | |
| | C2245 | Georgia, USA | *X. glabratus* | | EU177438 |
| | C2258 | Florida, USA | *X. glabratus* | | EU177439 |
| | C2339, CBS 121567* | South Carolina, USA | *X. glabratus* | | EU177440 |
| *R. montetyi* | C2221, CBS 463.94* | France | *P. cylindrus* | EU170283 | |
| | C2220, CBS 451.94 | Portugal | *P. cylindrus* | | EU177461 |
| *R. subalba* | C2368 | Georgia, USA | *X. glabratus* | | EU177441 |
| | C2388 | South Carolina, USA | *X. glabratus* | | EU177442 |
| | C2401, CBS 121568* | South Carolina, USA | *X. glabratus* | | EU177443 |
| *R. subfusca* | C2253 | Florida, USA | *X. glabratus* | EU170268 | |
| | C2335, CBS 121571* | South Carolina, USA | *X. glabratus* | | EU177450 |
| | C2352 | South Carolina, USA | *X. glabratus* | | EU177473 |
| | C2380 | Georgia, USA | *X. glabratus* | | EU177451 |
| *R. sulcati* | C2234, CBS 806.70* | British Columbia, Canada | *G. sulcatus* | EU170271 | EU177462 |
| *R. tritirachium* | C2222, CBS 726.69* | Pennsylvania, USA | *M. mali* | EU170273 | EU177464 |
| *Raffaelea* sp. | C1941 | South Carolina, USA | | EU170274 | |
| | C1943 | South Carolina, USA | | | EU177465 |
| | C2262 | South Carolina, USA | *X. glabratus* | | EU177466 |
| *O. huntii* | C583 | Michigan, USA | | | EU177469 |
| *O. ips* | C2308 | California, USA | *Orthotomicus erosus* | | EU177470 |
| *O. serpens* | C30, CBS 141.36* | Italy | | | EU177471 |
| *L. abietinum* | C1883 | Alaska, USA | | | EU177472 |

[a]Collection numbers are those of the senior author or the Centraalbureau voor Schimmelcultures (CBS). Isolates denoted with an asterisk are from the holotype.

### DNA sequencing and phylogenetic analyses

Isolates were grown on MYEA (2% Difco malt extract, 0.2% Difco yeast extract, and 1.5% agar) for 4–10 days at room temperature prior to DNA extraction. Mycelium was scraped from the surface, and DNA was extracted using PrepMan™ Ultra (Applied Biosystems, Foster City, CA). Amplification and sequencing of portions of the SSU (small subunit, 18S) rDNA and LSU (large subunit, 26S) rDNA were performed as described (Fraedrich et al. 2008). Primers for amplification and sequencing of the SSU rDNA included NS1, NS2, NS3, NS4, NS5, NS6, NS7, and NS8 (White et al. 1990) and SR1R and SR6 (Vilgalys & Hester 1990). The SR1R/SR6 products were cloned into pGEM T-easy vector (Promega Inc., Madison WI) and sequenced with flanking vector primers U (5'-TGTAAAACGACGGCCAGT-3') and R-1 (5'-CAGGAAACAGCTATGACC-3'), plus the internal primers NS2, NS3, NS5, and NS6. A portion of the LSU gene was amplified with primers LROR and LR5, and the PCR products were sequenced with primers LROR and LR3 (White et al. 1990). All sequences were generated at the Iowa State University DNA Sequencing and Synthesis Facility.

Phylogenetic analyses utilized SSU and LSU sequences available in GenBank as well as new sequences generated in this study (Table 1). Parsimony analysis and bootstrapping were carried out in PAUP 4.0b10 (Sinauer Associates, Sunderland, Massachusetts).

### Species descriptions

Cultures were grown on malt extract agar (MEA, 1% Difco malt extract and 1.5% agar) at 25 C in the dark. Growth at 5, 10, 15, 20, 25, 30 and 35 C was also determined on MEA. Cycloheximide tolerance was determined on MEA amended with 100 ppm cycloheximide, but the cycloheximide was dissolved in ethanol before adding to the autoclaved medium. Colors of cultures on MEA followed the nomenclature of Rayner (1970).

Representative cultures were deposited in the Centraalbureau voor Schimmelcultures, and herbarium specimens have been deposited in the U.S. National Fungus Collections (BPI).

## Results

Six filamentous fungal species were isolated from 39 adult female *X. glabratus*. Each of the six species was isolated in substantial numbers (greater than 300 colony forming units) from the surface-sterilized head of at least one beetle, suggesting that they were growing in the mycangium of the beetle, and most beetles yielded more than one fungal species. Each of the six fungal species tolerated cycloheximide, and they had SSU and LSU sequences similar to those of other *Ophiostoma*-like fungi that have been associated with ambrosia beetles (Figs. 1 and 2). All six species had small, inconspicuous conidiophores that produced conidia from their tips, with the conidiogenous cells proliferating percurrently, with no conspicuous scars. All produced blastospores, that is, conidia budded from conidia to form a conspicuous yeast phase on the surface of cultures.

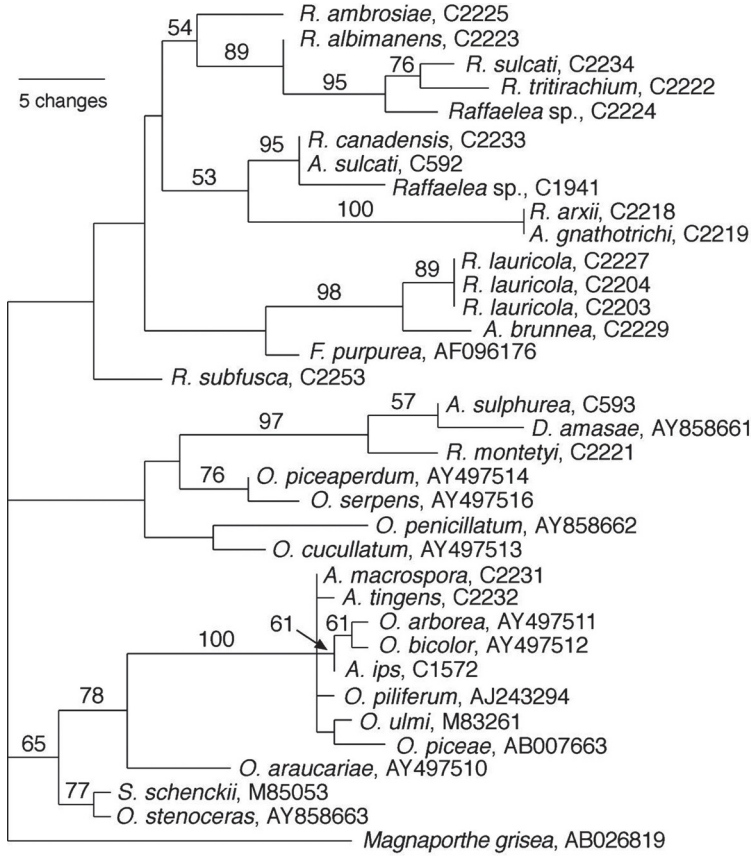


Fig. 1. One of two most-parsimonious trees of ambrosia beetle symbionts in the genera *Raffaelea*, *Ambrosiella*, and *Dryadomyces*, other *Ambrosiella* species associated with bark beetles, representative *Ophiostoma* species, *Fragosphaeria purpurea*, and *Sporothrix schenckii* based on sequences of SSU rDNA. The tree was rooted to *Magnaporthe grisea*. Isolate numbers (beginning with C) or GenBank accession numbers follow each taxon label. Consistency index = 0.5808, homoplasy index = 0.4192, retention index = 0.8238, and rescaled consistency index = 0.4785. Bootstrap values greater than 50% are shown above the branches.

The species isolated were distinguished from each other by mycelial morphology (Fig. 3), conidial morphology (Fig. 4), and analysis of LSU sequences (Fig. 2). The most commonly isolated species was *R. lauricola*, the cause of laurel wilt (Harrington et al. 2008), and another species was shown by LSU sequence to be *R. arxii*. Four species were undescribed species of *Raffaelea*. Detailed results of the isolations will be published elsewhere.

**Phylogenetic analyses**

Some of the species had SSU rDNA sequences with large introns that were unique to a single taxon, and these were eliminated from the analyses, leaving 1026 aligned characters, six of which were eliminated because of ambiguous alignment. Gaps were treated as a "fifth base," the characters were unordered, and all characters had equal weight. Of the 1020 characters, 919 were constant and 30 of the variable characters were parsimony uninformative, leaving 71 parsimony-informative characters. Two most parsimonious trees of 198 steps were generated from the SSU dataset (Fig. 1). Most of the major branches had little or no bootstrap support, and ambrosia beetle symbionts did not group into a single monophyletic group. However, *R. sulcati*, *R. tritirachium*, and an isolate submitted to CBS as *R. canadensis* (C2224) grouped together. Another group consisted of a culture from the holotype of *R. canadensis*, *A. sulcati*, and an unidentified *Raffaelea* species (Fig. 1). *Raffaelea montetyi*, *A. sulphurea*, and *D. amasae* also grouped together. The laurel wilt pathogen, *R. lauricola*, grouped with *A. brunnea*. *Raffaelea arxii* and *A. gnathotrichi* had an identical SSU sequence. *Ambrosiella tingens*, *A. macrospora*, and *A. ips*, which have been associated with bark beetles (Harrington 2005), grouped with *Ophiostoma arborea* (Olchow. & J. Reid) Yamaoka & M.J. Wingf., *O. bicolor* R.W. Davidson & D.E. Wells, *O. piliferum* (Fr.) Syd. & P. Syd., *O. ulmi* (Buisman) Nannf., and *O. piceae* (Münch) Syd. & P. Syd. (Fig. 1).

The partial LSU rDNA sequences were treated as in the SSU dataset, but no intron was detected. The LSU dataset had 561 aligned characters, 346 characters were constant, and 41 characters were parsimony-uninformative, leaving 174 parsimony-informative characters. A single most-parsimonious tree of 643 steps was found (Fig. 2). A weakly supported branch (53% bootstrap support) connected all of the sampled ambrosia beetle symbionts, including the four new species isolated from *X. glabratus*. Some of the *Ophiostoma* species with *Leptographium* Lagerb. & Melin anamorphs were sister to the group of ambrosia beetle symbionts, but this branch had only weak bootstrap support (55%). Two species (*R. arxii* and *A. gnathotrichi*) with identical SSU sequences (Fig. 1) had differing LSU sequences, but they grouped together with strong

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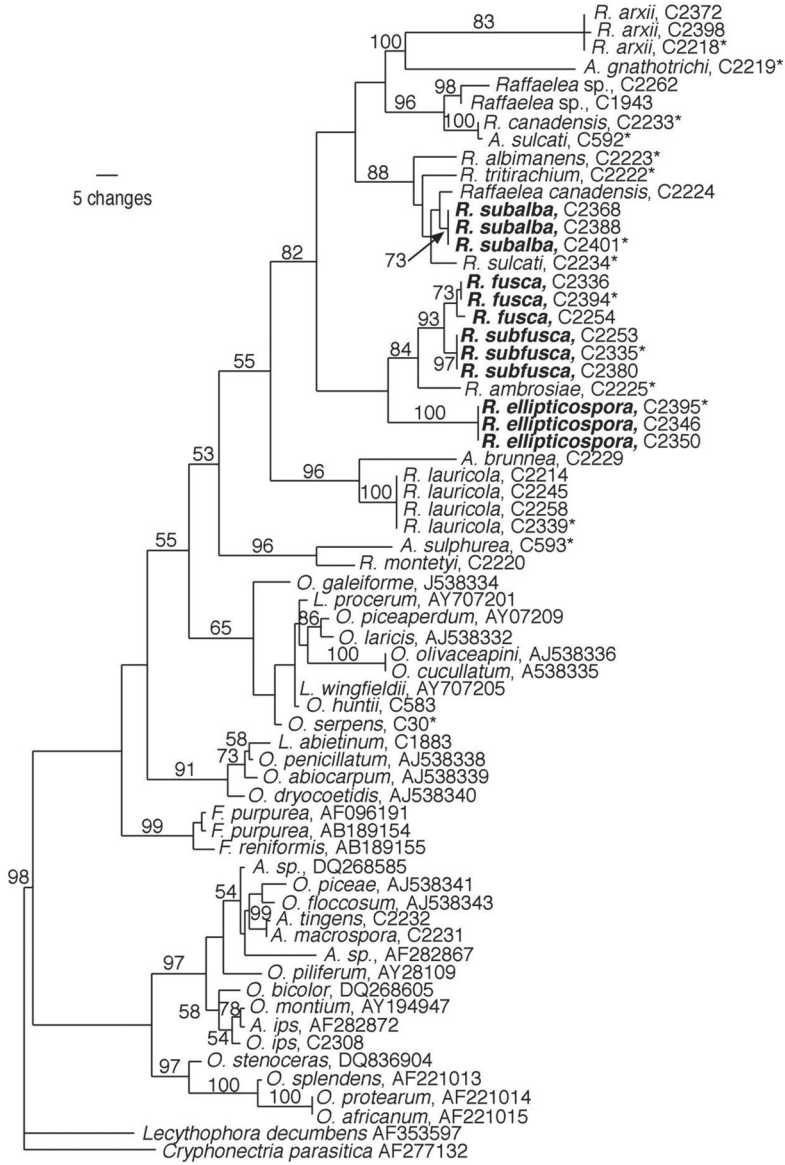
Fig. 2. The most-parsimonious tree of ambrosia beetle symbionts in the genera *Raffaelea* and *Ambrosiella*, other *Ambrosiella* species associated with bark beetles, and representative *Ophiostoma*, *Leptographium*, and *Fragosphaeria* species based on sequences of LSU rDNA. The tree was rooted to *Cryphonectria parasitica* and *Lecythophora decumbens,* allowing both the outgroup and ingroup taxa to collapse in a polytomy. Isolate numbers (beginning with C) or GenBank accession numbers follow each taxon label. Names of new species are in bold. New sequences of isolates from holotypes are followed by an asterisk. Consistency index = 0.4697, homoplasy index = 0.5303, retention index = 0.8253, and rescaled consistency index = 0.3876. Bootstrap values greater than 50% are shown above the branches.


5 changes
R. arxii, C2372
R. arxii, C2398
R. arxii, C2218*
A. gnathotrichi, C2219*
Raffaelea sp., C2262
Raffaelea sp., C1943
R. canadensis, C2233*
A. sulcati, C592*
R. albimanens, C2223*
R. tritirachium, C2222*
Raffaelea canadensis, C2224
R. subalba, C2368
R. subalba, C2388
R. subalba, C2401*
R. sulcati, C2234*
R. fusca, C2336
R. fusca, C2394*
R. fusca, C2254
R. subfusca, C2253
R. subfusca, C2335*
R. subfusca, C2380
R. ambrosiae, C2225*
R. ellipticospora, C2395*
R. ellipticospora, C2346
R. ellipticospora, C2350
A. brunnea, C2229
R. lauricola, C2214
R. lauricola, C2245
R. lauricola, C2258
R. lauricola, C2339*
A. sulphurea, C593*
R. montetyi, C2220
O. galeiforme, J538334
L. procerum, AY707201
O. piceaperdum, AY07209
O. laricis, AJ538332
O. olivaceapini, AJ538336
O. cucullatum, A538335
L. wingfieldii, AY707205
O. huntii, C583
O. serpens, C30*
L. abietinum, C1883
O. penicillatum, AJ538338
O. abiocarpum, AJ538339
O. dryocoetidis, AJ538340
F. purpurea, AF096191
F. purpurea, AB189154
F. reniformis, AB189155
A. sp., DQ268585
O. piceae, AJ538341
O. floccosum, AJ538343
A. tingens, C2232
A. macrospora, C2231
A. sp., AF282867
O. piliferum, AY28109
O. bicolor, DQ268605
O. montium, AY194947
A. ips, AF282872
O. ips, C2308
O. stenoceras, DQ836904
O. splendens, AF221013
O. protearum, AF221014
O. africanum, AF221015
Lecythophora decumbens AF353597
Cryphonectria parasitica AF277132

bootstrap support (Fig. 2). The holotypes of *R. canadensis* and *A. sulcati* had nearly identical LSU sequences, and *A. sulphurea* and *R. montetyi* grouped together, as did *R. lauricola* and *A. brunnea*. The *Ambrosiella* species associated with bark beetles (*Ambrosiella tingens*, *A. macrospora*, and *A. ips*) grouped with *O. ips* (Rumbold) Nannf., *O. piceae*, *O. piliferum,* and related *Ophiostoma* species (Fig. 2).

## Taxonomy

***Raffaelea*** Arx & Hennebert emend. T.C. Harr., Mycotaxon 104: 401. 2008

= *Dryadomyces* Gebhardt, Mycological Research 109: 693. 2005.

Type Species: *Raffaelea ambrosiae* Arx & Hennebert

Conidiophores single to aggregated in sporodochia, hyaline, unbranched or sparingly branched, one-celled to septate, producing conidia holoblastically. Conidiogenous cells proliferating percurrently or sympodially, leaving denticles, inconspicuous scars, or annellations. Conidia small, hyaline, elliptical to ovoid to globose, succession schizolytic, producing yeast-like growth through budding. Tolerating cycloheximide in culture. Associated with ambrosia beetles.

Comments — Conidiophores and conidia of *Raffaelea* species could fit the concept of *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr., a common anamorph of *Ophiostoma* species (Gebhardt & Oberwinkler 2005, Massoumi Alamouti et al. 2009, Upadhyay & Kendrick 1975, Zipfel et al. 2006). Past treatments have used the presence of sporodochia to distinguish *Raffaelea* from *Hyalorhinocladiella*, but Harrington et al. (2008) proposed that *Raffaelea* species are better distinguished by their symbiotic relationship with ambrosia beetles. That concept is followed here because it appears to distinguish an asexual, monophyletic group within *Ophiostoma* sensu lato (Fig. 2, Gebhardt et al. 2005, Kolarik & Hulcr 2009, Massoumi Alamouti et al. 2009). The generic names *Ambrosiella* and *Dryadomyces* have also been used for symbionts of ambrosia beetles related to *Ophiostoma*. However, the type species of *Ambrosiella* is closely related to *Ceratocystis* rather than *Ophiostoma*, and *Ambrosiella* species within the *Raffaelea* clade are here transferred to *Raffaelea*. *Dryadomyces,* initially separated by its large conidia and prominent scars on the conidiogenous cells (Gebhardt et al. 2005), is within the *Raffaelea* clade, and it is also transferred to *Raffaelea*.

### Four new *Raffaelea* species from *Xyleborus glabratus*

***Raffaelea subalba*** T.C. Harr., Aghayeva & Fraedrich, **sp. nov.** Figs. 3B, 4A–B

MycoBank 515291, GenBank EU177443

*Coloniae in agaro (MEA) post 10 dies ad 25 C, 25 mm diam, cremae-bubalinae. Conidia blastosporae, globosae vel ovatae, 4.5–5.0 × 3.5–4.0* μ*m. Socius cum* Xyleborus glabratus.

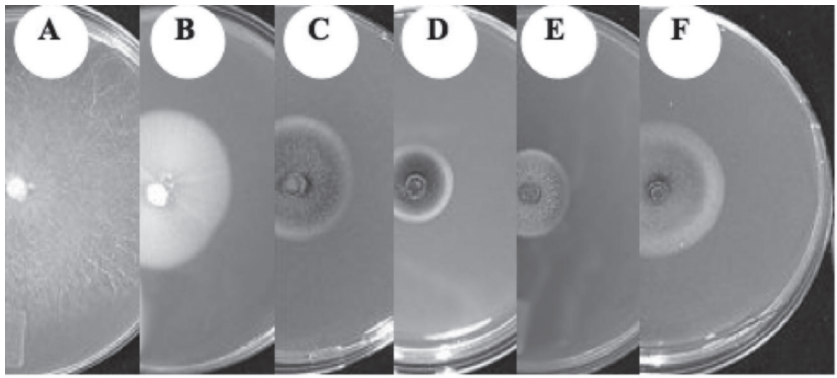



Fig. 3. Colony morphology after 11 days of *Raffaelea* species isolated from *Xyleborus glabratus* on 90 mm diameter plates of malt extract agar. A. *R. lauricola,* B. *R. subalba,* C. *R. ellipticospora,* D. *R. fusca,* E. *R. subfusca*, and F. *R. arxii*. Cultures are from the holotypes except for isolate C2372 of *R. arxii*.

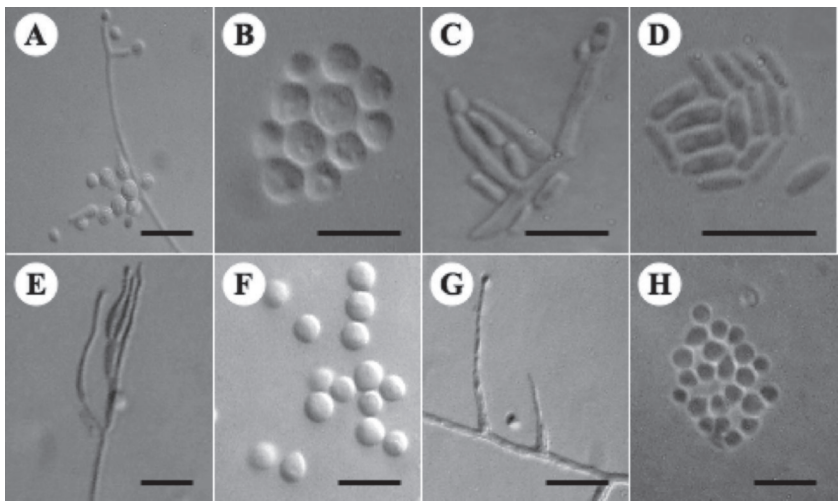



Fig. 4. Conidia and conidiophores of isolates from holotype specimens of four new *Raffaelea* species. A,B. *R. subalba;* C,D. *R. ellipticospora;* E,F. *R. fusca;* G,H. *R. subfusca*. Scale bars = 10 µm.

**Holotype**—**UNITED STATES**. **South Carolina**: Hunting Island State Park—*Xyleborus glabratus*, December 2006, S. Fraedrich, BPI 878184, from culture C2401 (= CBS 121568).

Colonies on malt agar attaining an average diameter of 25 mm in 10 days at 25 C in the dark. Trace growth at 10 C, no growth to 9 mm diameter at 35 C, maximum growth at 25 C. Mycelium at first smooth, cream-buff (19″d), aerial

hyphae scarce, usually smooth, later mucilaginous, margins of colony even, reverse without distinct color, aroma absent, 2 week old cultures cottony, rugose, buffy brown (17′′′i), with a yeasty odor. Conidiophores aseptate, discrete or fasciculate, terminal or arising from side branches, (9.5–)16.0–60(–120) × 1.5–2.0 μm, producing conidia holoblastically without leaving conspicuous scars or annellations. Conidia globose to ovate, sometimes pyriform, hyaline, thick-walled, (4.0–)4.5–5.0(–5.5) × (3.0–)3.5–4.0(–4.5) μm. Germinating conidia give rise to budding cells.

Cultures examined—**UNITED STATES. Georgia:** Jesup—*Xyleborus glabratus*, October 2006, S. Fraedrich, C2368; **South Carolina:** Hunting Island State Park—*X. glabratus*, October 2006, S. Fraedrich, C2388.

Comments — This species produces little pigment on MEA (Fig. 3). It was isolated from *X. glabratus* almost as frequently as *R. lauricola*, which grows at a much faster rate and produces much more mucilage (Harrington et al. 2008). In LSU sequence, *R. subalba* groups with *R. albimanens*, *R. tritirachium*, *R. sulcati*, and a South African isolate misidentified as *R. canadensis* (Fig. 2).

***Raffaelea ellipticospora*** T.C. Harr., Aghayeva & Fraedrich, **sp. nov.** Figs. 3C, 4C–D

MycoBank 515292, GenBank EU177446

*Coloniae in agaro (MEA) post 10 dies ad 25 C, 18 mm diam, brunneolae-olivaceae. Conidia blastosporae, ellipticae vel oblongatae, 5.0–5.5 × 1.0–2.0 μm. Socius cum* Xyleborus glabratus.

**Holotype—UNITED STATES. South Carolina**: Hunting Island State Park—*Xyleborus glabratus*, December 2006, S. Fraedrich, BPI 878185, from culture C2395 (= CBS 121569).

Colonies on malt extract agar attaining an average diameter of 18 mm in 10 days at 25 C in the dark. No growth at 10 or 35 C, maximum growth at 25 C. Mycelium brown to olivaceous (23m), darker in the center, indistinct white near the edges, edges even, reverse indistinct gray to brownish, aroma absent. Two-week-old cultures gray-brown or dark mouse-gray (15′′′′k), with yeasty odor, producing sporodochia reduced to discrete fascicles. Hyphae branched, smooth, hyaline, septate, aerial hyphae scarce. Conidiophores micronematous, mononematous, erect, cylindrical, fasciculate, hyaline, septate, (17–)30–60(–80) × 1.5–2.0(–2.5) μm. Conidia produced singly, ellipsoid to oblong to pyriform, hyaline, (4.0–)5.0–5.5(–6.0) × 1.0–2.0 μm, sometimes larger, 6.5–9.0 × 2.5–4.0 μm.

Cultures examined—**UNITED STATES. South Carolina**: Hunting Island State Park—*Xyleborus glabratus*, June 2006, S. Fraedrich, C2346. Hunting Island State Park—*X. glabratus*, June 2006, S. Fraedrich, C2350.

Comments — This species is distinguished from other species isolated from *X. glabratus* by its elliptical spores and unique LSU sequence (Fig. 2).

***Raffaelea fusca*** T.C. Harr., Aghayeva & Fraedrich, **sp. nov.** Figs. 3D, 4E–F

MycoBank 515293, GenBank EU177449

*Coloniae in agaro (MEA) post 10 dies ad 25 C, 13 mm diam, fuscae-olivaceae. Conidia blastosporae, oblongatae vel ovatae, 4.0–4.5 × 4.0–4.5 µm. Socius cum* Xyleborus glabratus.

**Holotype—UNITED STATES. South Carolina**: Hunting Island State Park—*Xyleborus glabratus*, December 2006, S. Fraedrich, BPI 878186, from culture C2394 (= CBS 121570).

Colonies on malt extract agar attaining a diameter of 13 mm in 10 days at 25 C in the dark. Trace to no growth at 10 C and no growth at 35 C, maximum growth at 25 to 30 C. Mycelium dark brown to brownish-olive (19″m) in the center, with indistinct white border, edges even, later mucilaginous, reverse gray to brownish, aroma absent. Hyphae branched, smooth, hyaline, septate, aerial hyphae scarce. Two-week-old cultures develop mat-like mycelia with concentric rings, fuscous black (13‴m) to mouse gray (15⁗) in the center, with yeasty odor, sporodochia reduced to discrete fascicles. Conidiophores micronematous, mononematous, erect, cylindrical, fasciculate, hyaline, aseptate, scattered, (13.0–)16.0–26.5 × 1.0–1.5(–2.0) µm. Conidia produced singly, ovate to obovoid, sometimes pyriform, hyaline, (3.5–)4.0–5.0(–6.5) × (3.5–)4.0–4.5(–5.0) µm.

Cultures examined—**UNITED STATES. Florida**: Fort George Island—*X. glabratus*, December 2005, S. Fraedrich, C2254. **South Carolina**: Hunting Island State Park—*X. glabratus*, June 2006, S. Fraedrich, C2336.

Comments — This species produces conidia similar to those of *R. subfusca*, but cultures of *R. fusca* on MEA produce a darker pigmentation (Fig. 3). The LSU sequences of *R. fusca* and *R. subfusca* are also similar, and both are similar to that of *R. ambrosiae* (Fig. 2).

***Raffaelea subfusca*** T.C. Harr., Aghayeva & Fraedrich, **sp. nov**. Figs. 3E, 4G–H

MycoBank 515294, GenBank EU177450

*Coloniae in agaro (MEA) post 10 dies ad 25 C, 13 mm diam, pallidae subfuscae-olivaceae. Conidia blastosporae, obovatae vel ovatae, 4.0–5.0 × 3.0–4.0 µm. Socius cum* Xyleborus glabratus.

**Holotype—UNITED STATES. South Carolina**: Hunting Island State Park—*Xyleborus glabratus*, June 2006, S. Fraedrich, BPI 878187, from culture C2335 (= CBS 121571).

Colonies on malt extract agar attaining an average diameter of 13 mm in 10 days at 25 C in the dark. Trace of growth at 10 C and 8 to 12 mm diameter at 35 C, maximum growth at 25–30 C. Mycelium light olivaceous (21‴m), darker in the center, indistinct-white near the edges, edges even, reverse indistinct

gray to brownish, aroma absent. Two-week-old cultures grayish-sepia (17′′′′′i) at the edges and mouse-gray (15′′′′′) in the center, wrinkled, with faint concentric circles, producing sporodochia reduced to discrete fascicles, aerial hyphae scarce. Hyphae branched, smooth, hyaline, septate. Conidiophores micronematous, mononematous, erect, cylindrical, fasciculate, hyaline, septate, scattered, (5–)12–38(–50) × 1.0–1.5(–2.0) µm. Conidia produced singly, ovate to obovoid, sometimes pyriform, (3.5–)4.0–5.0 × (2.5–)3.0–4.0(–5.0) µm.

Cultures examined—**UNITED STATES. Georgia**: jesup—*X. glabratus*, October 2006, S. Fraedrich, C2380; **South Carolina**: Hunting Island State Park—*X. glabratus*, June 2006, S. Fraedrich, C2352.

Comments — This species produces conidia similar to those of *R. fusca*, but cultures of *R. subfusca* on MEA produce a lighter pigmentation (Fig. 3), and *R. fusca* fails to grow at 35 C. The LSU sequence of *R. subfusca* and *R. fusca* are also similar (Fig. 2).

### Other *Raffaelea* species

***Raffaelea albimanens*** D.B. Scott & J.W. du Toit, Trans. Br. Mycol. Soc. 55: 181. 1970.

Comments — This species is related to *R. sulcati* and *R. tritirachium* based on DNA sequence analyses (Figs. 1 and 2, Massoumi Alamouti et al. 2009). It was described from *Platypus externedentatus* Fairm. in South Africa (Scott & du Toit 1970).

***Raffaelea amasae*** (Gebhardt) T.C. Harr., **comb. nov.**

MycoBank 515295

≡ *Dryadomyces amasae* Gebhardt, Mycolog. Res. 109: 693. 2005.

Comments — The SSU trees (Fig 1, Gebhardt et al. 2005) and the multigene phylogeny by Massoumi Alamouti et al. (2009) place *R. amasae* within the *Raffaelea* clade near *R. montetyi* and *A. sulphurea*. *R. amasae* is a symbiont of *Amasa concitatus* Wood & Bright (Gebhardt et al. 2005). The somewhat large conidia and prominent scars on the conidiogenous cells at the point of conidial dehiscence are not considered sufficiently distinct to warrant the monotypic genus *Dryadomyces* (Harrington et al. 2008).

***Raffaelea ambrosiae*** Arx & Hennebert, Mycopathol. Mycol. Appl. 25: 310. 1965.

Comments — This type species for the genus *Raffaelea* (Arx & Hennebert 1964) groups near two of the new species from *X. glabratus* in the LSU tree (Fig. 2) and within *Raffaelea* by the multigene phylogeny by Massoumi Alamouti et al. (2009). It has been associated with species of *Platypus* in Europe and the USA (Batra 1968).

***Raffaelea arxii*** D.B. Scott & J.W. du Toit, Trans. Br. Mycol. Soc. 55: 184. 1970.

Comments — The isolate from the holotype (C2218 = CBS 273.70) is near *A. gnathotrichi* in the SSU (Fig. 1), the LSU (Fig. 2), and the multigene trees (Massoumi Alamouti et al. 2009). Isolates with the same LSU sequence were obtained from *X. glabratus*, and *R. arxii* was originally described from an ambrosia beetle of the same genus, *X. torquatus* Eichh., in South Africa.

***Raffaelea brunnea*** (L.R. Batra) T.C. Harr., **comb. nov.**

MycoBank 515296

≡ *Monilia brunnea* Verrall, J. Agr. Res. 66: 142. 1943, nom. illegit. [non J.C. Gilman & E.V. Abbott 1927].

≡ *Ambrosiella brunnea* L.R. Batra, Mycologia 59: 980. 1968 ("1967").

Comments — This species is near *R. lauricola* based on DNA sequences (Figs. 1 and 2; Massoumi Alamouti et al. 2009). It was associated with species of *Monarthrum* on *Quercus* in the USA (Batra 1968).

***Raffaelea canadensis*** L.R. Batra, Mycologia 59: 1010. 1968 ("1967").

≡ *Tuberculariella ambrosiae* A. Funk, Canad. J. Bot. 43: 929. 1965.

= *Ambrosiella sulcati* A. Funk, Canad. J. Bot. 48: 1445. 1970.

Comments — In transferring *Tuberculariella ambrosiae* to *Raffaelea*, Batra (1968) introduced the replacement epithet *canadensis* to avoid creating a homonym of the earlier name *Raffaelea ambrosiae* Arx & Hennebert. Isolate C2233 (= CBS 168.66) from the holotype of *T. ambrosiae* has the same SSU sequence (Fig. 1) as isolate C592 (= CBS 805.70), the holotype of *A. sulcati*, and their LSU sequences are nearly identical (Fig. 2). The multigene phylogeny by Massoumi Alamouti et al. (2009) also shows nearly identical sequences for these two isolates. Descriptions of *A. sulcati* (Funk 1970) and *R. canadensis* (Batra 1968, Funk 1965) are similar, and the two species are considered synonyms. Isolate C2224 from South Africa was deposited in CBS (CBS 326.70) by Scott & du Toit (1970) as *R. canadensis*, but SSU (Fig. 1) and LSU (Fig. 2) sequences place this isolate near *R. sulcati*, and it is considered to be a misidentified isolate. *Raffaelea sulcati* is a species distinct from *A. sulcati* (Funk 1970). *Raffaelea canadensis* has been associated with *Platypus wilsoni* Swaine and *Gnathotrichus sulcatus* Lec. (as *A. sulcati)* in *Pseudotsuga menziesii* (Mirb.) Franco (Funk 1965, 1970).

***Raffaelea gnathotrichi*** (L.R. Batra) T.C. Harr., **comb. nov.**

MycoBank 515297

≡ *Ambrosiella gnathotrichi* L.R. Batra, Mycologia 59: 986. 1968 ("1967").

Comments — This species appears to be related to *R. arxii* by sequence analysis (Figs. 1 and 2, Massoumi Alamouti et al. 2009). Batra (1968) associated *R. gnathotrichi* with *Gnathotrichus retusus* Lec. on conifers in Colorado.

***Raffaelea lauricola*** T.C. Harr., Fraedrich & Aghayeva, Mycotaxon 104: 401. 2008.

Comments — This lethal pathogen of redbay and other members of the *Lauraceae* is probably from Asia, brought to the USA in mycangia of *X. glabratus* (Harrington et al. 2008).

***Raffaelea montetyi*** M. Morelet, Ann. Soc. Sci. Nat. Arch. Toulon Var 50: 189. 1998.

Comments — This associate of *Platypus cylindrus* Fab. in Europe (Morelet 1998) is related to *A. sulphurea* and *R. amasae* based on SSU analysis (Fig. 1) and a multigene phylogeny (Massoumi Alamouti et al. 2009).

***Raffaelea quercivora*** Kubono & Shin. Ito, Mycoscience 43: 256. 2002.

Comments — At the time of these analyses, no DNA sequence of this symbiont of *Platypus quercivorus* Murayama had been deposited, but a partial LSU sequence is similar to that of *R. montetyi* (Harrington unpublished).

***Raffaelea quercus-mongolicae*** K.H. Kim, Y.J. Choi, & H.D. Shin, Mycotaxon 110: 193. 2009.

Comments — This recently described species is closely related to *R. quercivora*, and the two symbionts are associated with closely related species of *Platypus* (Kim et al. 2009).

***Raffaelea santoroi*** Guerrero, Revt. Invest. Agropec., Sér. 5, 3: 100. 1966.

Comments — A multigene phylogeny (Massoumi Alamouti et al. 2009) placed this species near *R. tritirachium*. It was originally isolated from a bore hole of a *Platypus* sp. in Argentina (Guerrero 1966).

***Raffaelea scolytodis*** M. Kolarik, Mycol. Res. 113: 50. 2009.

Comments — Analysis of SSU and LSU sequences placed *R. scolytodis* among other *Raffaelea* species (Kolarik & Hulcr 2009). It was associated with *Scolytodes unipunctatus* Wood & Bright, the only ambrosia beetle in the genus (Hulcr et al. 2007).

***Raffaelea sulcati*** A. Funk, Canad. J. Bot. 48: 1447. 1970.

Comments — The LSU sequence (Fig. 2) of a culture from the holotype confirms placement of this species in *Raffaelea*. It was associated with *Gnathotrichus sulcatus* in *Pseudotsuga menziesii*. Funk (1970) described *Ambrosiella sulcati* at the same time as *R. sulcati*, distinguishing the former by monilioid chains of conidia, and the latter by sympodial proliferation of conidiogenous cells. *Ambrosiella sulcati* is treated above as a synonym of *R. canadensis*.

***Raffaelea sulphurea*** (L.R. Batra) T.C. Harr., **comb. nov.**

MycoBank 515298

≡ *Ambrosiella sulphurea* L.R. Batra, Mycologia 59: 992. 1968 ("1967").

Comments — The LSU sequence (Fig. 2) of a culture from the holotype is close to that of *R. montetyi*, and *R. amasae* is also related to these two species based on SSU (Fig. 1) and multigene analyses (Massoumi Alamouti et al. 2009). It was described (Batra 1968) from *X. saxeseni* Ratzeb.

***Raffaelea tritirachium*** L.R. Batra, Mycologia 59: 1013. 1968 ("1967").

Comments — In DNA sequence, *R. tritirachium* appears near *R. albimanens*, *R. sulcati*, *R. santoroi* and one of the new species from *X. glabratus* (Figs. 1 and 2, Massoumi Alamouti et al. 2009). Batra (1968) considered *R. tritirachium* a contaminant in galleries of *Monarthrum mali* (Fitch), an ambrosia beetle more commonly associated with *R. brunnea*.

### Uncertain or excluded species of *Raffaelea*

***Fusarium barbatum*** Ellis & Everh., J. Mycol. 4: 45. 1888.

≡ *Raffaelea barbata* (Ellis & Everh.) D. Hawksw., Bull. Br. Mus. Nat. Hist. 6: 272. 1979.

Comments —Hawksworth (1979) transferred *F. barbatum* to *Raffaelea* based on production of sporodochia, which were found on the lichen *Usnea barbata*. But the fungus appears to be properly placed among anamorphic *Hypocreales*, i.e., *Fusarium barbatum*.

***Pseudallescheria boydii*** (Shear) McGinnis, A.A. Padhye & Ajello, Mycotaxon 14: 97. 1982.

= *Raffaelea castellanii* (Pinoy) de Hoog, Stud. Mycol. 7: 44. 1974.

Comments —This human pathogen (de Hoog 1974) is properly placed in the *Microascales*.

***Raffaelea hennebertii*** D.B. Scott & J.W. du Toit, Trans. Br. Mycol. Soc. 55: 183. 1970.

Comments — Scott & du Toit (1970) described *R. hennebertii* from *Platypus externedentatus* in South Africa, and their description and illustration are consistent with a species of *Raffaelea*. However, an isolate from the holotype (CBS 272.70) was found to have an SSU sequence near *Melanospora* (*Melanosporales*) by Jones & Blackwell (1998). Further work is needed to be sure the isolate is not a contaminant.

***Raffaelea variabilis*** B. Sutton, Antonie van Leeuwenhoek 41: 179. 1975.

Comments —This species was isolated from the plant *Lannea grandis* (Dennst.) Engl. in Malaysia and was not associated with an ambrosia beetle (Sutton 1975). Thus, it does not ecologically fit the concept of *Raffaelea* presented here.

### *Ambrosiella* species

***Ambrosiella*** Brader ex Arx & Hennebert **emend.** T.C. Harr.

Type Species—*Ambrosiella xylebori* Brader ex Arx & Hennebert

Conidiophores single to aggregated in sporodochia, hyaline, unbranched or sparingly branched, one-celled to septate, producing terminal aleurioconidia or chains of conidia from phialides. Sensitive to cycloheximide in culture. Related to species of *Ceratocystis*. Associated with ambrosia beetles.

Comments — The genus is herein restricted to ambrosia beetle symbionts producing conidia from phialides and related to the genus *Ceratocystis*. Five species are recognized in *Ambrosiella* sensu stricto. All are known symbionts of ambrosia beetles and produce conidia from phialides by ring-wall building (Gebhardt et al. 2005). Phylogenetic analyses place four of the species within the genus *Ceratocystis*, though the *Ambrosiella* species do not appear to be a monophyletic group (Harrington 2009, Kolarik & Hulcr 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009). Sexual states for *Ambrosiella* species are not known. The genus name *Thielaviopsis* has been proposed for anamorphs of *Ceratocystis* species (Paulin-Mahady et al. 2002), but *Ambrosiella* is retained here for *Thielaviopsis*-like species associated with ambrosia beetles.

***Ambrosiella beaveri*** Six, De Beer & W.D. Stone, Antonie van Leeuwenhoek 96: 23. 2009.

Comments — This recently-described species is closely related to *A. xylebori* and *A. hartigii* within the *Ceratocystis* group based on LSU and β-tubulin analyses (Six et al. 2009). It was recently described from the ambrosia beetle *Xylosandrus mutilatus* (Blandford) (Six et al. 2009).

***Ambrosiella ferruginea*** L.R. Batra, Mycologia 59: 980. 1968 ("1967").

≡ *Monilia ferruginea* Math.-Käärik, Meddel. Statens Skogsforskningsinstitut 43(4): 57. 1954, non. illegit. [non Pers. 1822]

Comments — Sequence analyses place this associate of *Trypodendron* and *Xyloterus signatus* Fabr. in *Ceratocystis*, but it is not clear whether *A. xylebori* and *A. hartigii* are its nearest relatives (Harrington 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009).

***Ambrosiella hartigii*** L.R. Batra, Mycologia 59: 998. 1968 ("1967").

Comments — This species is close to *A. xylebori*, the type species of the genus, based on sequences of several genes (Harrington 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009). It has been associated with *Xyleborus dispar* (Fabr.) and *Xylosandrus germanus* (Blandford) (Batra 1968).

***Ambrosiella trypodendri*** (L.R. Batra) T.C. Harr., **comb. nov.**

MycoBank 515299

≡ *Phialophoropsis trypodendri* L.R. Batra, Mycologia 59: 1008. 1968 ("1967").

Comments — Batra's (1968) description of this associate of *Trypodendron scabricollis* (Lec.) states that the conidia are thick-walled phialospores, and his illustrations show spores typical of other *Ambrosiella* species related to *Ceratocystis*, such as *A. hartigii*. No culture or DNA sequence was available for study.

***Ambrosiella xylebori*** Brader ex Arx & Hennebert, Mycopath. Mycol. Appl. 25: 314. 1965.

Comments — This species, the type species for the genus, has been associated with *Xylosandrus compactus* Eichh. and *Corthylus columbianus* (Hopkins) (Arx & Hennebert 1965, Batra 1968). The DNA sequences of *A. xylebori* are close to those of *A. hartigii* and somewhat near *Ceratocystis adiposa* (Harrington 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009).

### New combinations in *Hyalorhinocladiella* from *Ambrosiella*

Three species of *Ambrosiella* that are fed upon by bark beetles are excluded from *Raffaelea* and *Ambrosiella* by DNA sequence analyses (Figs. 1 and 2). They form sporodochia, but their simple conidiophores otherwise fit in Upadhyay & Kendrick's (1975) concept of *Hyalorhinocladiella* (*Rhinocladiella*-like, but lacking pigmentation). *Hyalorhinocladiella* species are distinguished from *Sporothrix* by the lack of prominent denticles on the conidiogenous cells (de Hoog 1993).

***Hyalorhinocladiella ips*** (J.G. Leach, L.W. Orr, & C.M. Chr.) T.C. Harr., **comb. nov.**

MycoBank 515302

≡ *Tuberculariella ips* J.G. Leach, L.W. Orr, & C.M. Chr., J. Agr. Res. 49: 335. 1934.

≡ *Ambrosiella ips* (J.G. Leach, L.W. Orr, & C.M. Chr.) L.R. Batra, Mycologia 59: 980. 1968 ("1967").

Comments — Sequence analyses place this species among *Ophiostoma* species with *Hyalorhinocladiella* anamorphs, especially the species with box-shaped ascospores, such as *O. ips*, *O. bicolor*, and *O. montium* (Figs. 1 and 2, Massoumi Alamouti et al. 2009). *Hyalorhinocladiella ips* forms sporodochia in galleries, an adaptation for fungal feeding by insects, but it is fed upon by bark beetles in the genus *Ips*, not by ambrosia beetles (Harrington 2005).

***Hyalorhinocladiella macrospora*** (Francke-Grosm.) T.C. Harr., **comb. nov.**

MycoBank 515303

≡ *Trichosporium tingens* var. *macrosporum* Francke-Grosm., Meddel. Statens Skogs-forskningsinstitut 41(6): 27. 1953 ("1952").

≡ *Ambrosiella macrospora* (Francke-Grosm.) L.R. Batra, Mycologia 59: 980, 1968 ("1967").

Comments — This species was originally described as a variety of *T. tingens*, distinguished by its large conidia. *Hyalorhinocladiella macrospora* has been associated with the mycophagous bark beetle *Ips acuminatus* (Batra 1968, Harrington 2005). The DNA sequences of *H. macrospora* and *H. tingens* are similar (Figs. 1 and 2, Massoumi Alamouti et al. 2009). Both *H. macrospora* and *H. tingens* produce sporodochia, an adaptation for being fed upon by beetles (Harrington 2005). These species may be related in DNA sequence to *Ophiostoma* species with *Pesotum* anamorphs, but more detailed analyses are needed (Figs. 1 and 2, Massoumi Alamouti et al. 2009). Batra (1968) reported that the conidia of *H. macrospora* are formed blastically, consistent with *Hyalorhinocladiella*.

***Hyalorhinocladiella tingens*** (Lagerb. & Melin) T.C. Harr., **comb. nov.**

MycoBank 515304

≡ *Trichosporium tingens* Lagerb. & Melin, Svenska Skogsvårdsför. Tidskr. Yr., 1927: 215. 1927.

≡ *Ambrosiella tingens* (Lagerb. & Melin) L.R. Batra, Mycologia 59: 980, 1968 ("1967").

Comments — This species and *H. macrospora*, which was originally described as a variety of *tingens*, may be related in DNA sequence to *Ophiostoma* species with *Pesotum* anamorphs (Figs. 1 and 2, Massoumi Alamouti et al. 2009). It has been associated with mycophagous bark beetles in the genera *Ips* and *Tomicus* in Europe (Batra 1968, Harrington 2005).

## Discussion

The asexual, cycloheximide-tolerant symbionts of ambrosia beetles occur in a monophyletic clade within the genus *Ophiostoma*, and *Raffaelea* is proposed as the proper asexual genus for members of this group. The SSU rDNA trees presented by Kolarik & Hulcr (2009) and Gebhardt et al. (2005) both support the monophyletic nature of *Raffaelea*. Our analysis of SSU rDNA does not support the monophyly of *Raffaelea*, but our LSU rDNA analysis does. The multigene analysis of SSU, 5.8S, and LSU rDNA and β-tubulin (Massoumi Alamouti et al. 2009) also infers that the genus *Raffaelea* as proposed here is a monophyletic group. Massoumi Alamouti et al. (2009) found two subclades within *Raffaelea* to have bootstrap support and suggested that these should be recognized as separate genera. However, no phenotypic character distinguishes these two subclades.

Many groups of ascomycetes and basidiomycetes have evolved adaptations for grazing by bark and ambrosia beetles, most notably the aggregation of conidiophores or basidia in dense sporodochia or hymenia within larval

galleries or pupal chambers (Harrington 2005). The *Ophiostomataceae* are believed to have evolved about the time of the rise of conifer bark beetles (Farrell et al. 2001), and *Ophiostoma* species are among the most common associates of conifer bark beetles (Harrington 2005). Ambrosia beetles evolved from bark beetles in at least seven separate events (Farrell et al. 2001), and it is not surprising that most of the symbionts of ambrosia beetles are found in the *Ophiostomataceae*. It is surprising to find, however, that all the asexual symbionts in the *Ophiostoma* clade that are associated with ambrosia beetles may have evolved from a single ancestor. The ancestor of *Raffaelea* may have been uniquely successful in both serving as food for ambrosia beetles (sporodochial phase) and for reproducing in the mycangia of ambrosia beetles (yeast phase). Within *Ceratocystis*, adaptation for ambrosia beetle symbiosis may have arisen at least twice because *A. ferruginea* appears to have arisen as a symbiont separately from the *A. hartigii*, *A. xylebori*, and *A. beaveri* complex (Harrington 2009, Massoumi Alamouti et al. 2009, Paulin-Mahady et al. 2002, Six et al. 2009).

The SSU rDNA analysis by Gebhardt et al. (2005) and our LSU rDNA tree have two groups of *Ophiostoma* species with *Leptographium* anamorphs basal to the *Raffaelea* clade. The combined dataset of Massoumi Alamouti et al. (2009) groups the *Ophiostoma* species with *Leptographium* anamorphs as sister to *Raffaelea*. The SSU rDNA analysis of Kolarik & Hulcr (2009) has *Fragosphaeria purpurea* Shear as closer to the *Raffaelea* species than the *Ophiostoma* species with *Leptographium* anamorphs, but our LSU rDNA analysis has *F. purpurea* and *F. reniformis* (Sacc. & Therry) Malloch & Cain as basal to the clades with *Leptographium* anamorphs and *Raffaelea*. *Raffaelea* species produce conidia blastically, usually without prominent denticles, consistent with the conidiogenous cells of *Leptographium* anamorphs and the anamorph of *F. purpurea* (Shear 1923, Zipfel et al. 2006). Ecologically, *Raffaelea* species appear to be tied more closely to *Ophiostoma* species with *Leptographium* anamorphs, which are mostly associates of conifer bark beetles, than to *Fragosphaeria* species, which are considered to be saprophytes on wood (Malloch & Cain 1970, Shear 1923).

Zipfel et al. (2006) proposed that *Ophiostoma* species with *Leptographium* anamorphs be recognized as a separate genus, *Grosmannia* Goid. Their analysis of combined LSU rDNA and β-tubulin sequences showed good support for *Grosmannia* as a monophyletic group, as did analysis of the combined dataset of Massoumi Alamouti et al. (2009). However, our LSU rDNA analysis and the SSU analyses by Gebhardt et al. (2005) and Kolarik & Hulcr (2009) show two or more distinct clades within *Grosmannia* that do not form a monophyletic group. Inclusion of different taxa, limited taxon sampling, and relatively few protein-coding genes probably are the causes of the discrepancies among

the studies. For instance, the presence or absence of *Fragosphaeria* species appears to affect the topology of the trees. The proposal by Zipfel et al. (2006) to recognize *Grosmannia* may prove to have merit when more taxa and genes are included in the analyses, but the currently available phylogenetic analyses are ambiguous in determining if all *Ophiostoma* species with *Leptographium* anamorphs form a monophyletic group.

Three species of *Ambrosiella* appeared more closely related to other species of *Ophiostoma* than to *Raffaelea* species or *Ophiostoma* species with *Leptographium* anamorphs. These species resemble *Hyalorhinocladiella* species, except for their aggregation of conidiophores into sporodochia. Each of these species is fed upon by mycophagous bark beetles (Harrington 2005). Although *H. tingens* and *H. macrospora* appear to be closely related by our LSU rDNA analysis, *H. ips* appears to be more closely related to *O. ips* and *O. montium*, another *Ophiostoma* species fed upon by mycophagous bark beetles (Harrington 2005).

It has generally been accepted that one or a few fungal species are associated with a particular ambrosia beetle species (Batra 1963, Funk 1970), but six species of *Raffaelea* were isolated from *Xyleborus glabratus* in this study. The serial dilution plating technique that we used (Harrington 1992) and the use of cycloheximide in the isolation medium facilitate better recovery of *Raffaelea* species than have other isolation techniques used in the past. As better isolation techniques and DNA sequencing are applied, it is likely to be found that many ambrosia beetles are associated with numerous fungal symbionts.

Reduced morphology of *Raffaelea* species and their highly pleomorphic nature in culture have made it difficult to distinguish species. Some of the six species isolated from *X. glabratus* changed dramatically in culture over time, after storage, and on different media. Thus far, the LSU rDNA sequences appear useful in distinguishing species of *Raffaelea*. Unfortunately, the more variable internal transcribed spacer regions of rDNA are difficult to amplify in some of the *Raffaelea* species, such as *R. lauricola* (Fraedrich et al. 2008). The SSU sequences do not show sufficient variation to distinguish all of the known species of *Raffaelea*.

It is assumed that the six *Raffaelea* species isolated from *X. glabratus* were brought to the USA from Asia with the single introduction of *X. glabratus* to the Savannah, Georgia area (Fraedrich et al. 2008). It is possible that *X. glabratus* has acquired symbionts from other ambrosia beetle species since its arrival in the USA. However, Harrington & Fraedrich (unpublished) have only isolated a true *Ambrosiella* species from *Xylosandrus crassiusculus*, the most common ambrosia beetle competing with *Xyleborus glabratus* in stems of diseased redbay (Fraedrich et al. 2008). If *X. glabratus* brought six *Raffaelea* species with it from

a single introduction of the beetle, then even more species of *Raffaelea* may be associated with this beetle in Asia.

It is also common to find mycelial yeasts, *Pichia* species, and species of *Ophiostoma*, *Pesotum*, *Leptographium, Fusarium*, and other filamentous ascomycetes casually associated with ambrosia beetles, usually as secondary colonizers of galleries or superficial contaminants of adults (Batra 1963, 1968). Of the fungi that have been tightly associated with ambrosia beetles, that is, species isolated from mycangia and ambrosial growth in galleries, the majority have been species of *Raffaelea* as recognized here. Considering that a single, introduced population of *X. glabratus* carries six species of *Raffaelea* in its mycangia, that there appears to be some level of specificity, and that there are about 3400 described species of ambrosia beetles (Farrell et al. 2001), there may be many hundreds of species of *Raffaelea* awaiting description.

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