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Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles

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ABSTRACT

Most 'ambrosia' fungi are members of a heterogeneous group of ophiostomatoids that includes the anamorph genera *Ambrosiella*, *Raffaelea* and *Dryadomyces*. The taxonomy of these fungi based on morphological features has been complicated by these features being poorly descriptive and having evolved convergently. In this work we report maximum parsimony and Bayesian phylogenetic analysis of a multigene dataset (nSSU rDNA, nLSU rDNA and β-tubulin gene) from sixty-seven taxa that include members of genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* and a diverse set of ophiostomatoid relatives. We discuss the phylogenetic status of genus *Ambrosiella* and its relationships with representatives of *Ophiostomatales* teleomorph and anamorph genera. Our analysis shows that ten of the thirteen species that had been assigned to the genus *Ambrosiella* are related to the teleomorph genera *Grosmannia* or *Ophiostoma*, within the *Ophiostomatales*. The multigene analysis and expanded taxon samplings provide a higher resolution for the species phylogeny and clarify detailed relationships between *Ambrosiella* associates of ambrosia and bark beetles and the closely related species of genera *Raffaelea* and *Dryadomyces*. We discuss difficulties in using the morphology of conidiophores and the mode of conidiogenesis to re-define the phylogenetic classification of *Ambrosiella* species. Finally, we report a correlation between the molecular classification of *Ophiostomatales*-related species of *Ambrosiella* and *Raffaelea* and their ecological niches.

Introduction

Bark and ambrosia beetles are weevils (*Coleoptera*: *Curculionidae*) in the subfamilies *Scolytinae* and *Platypodinae* (Farrell *et al.* 2001; Marvaldi *et al.* 2002). They spend part of their life cycles in galleries that they mine under the bark (scolytid bark beetles) or in the wood of trees (scolytid and platypodid ambrosia beetles); and they vector diverse fungi that colonise the wood (Batra 1966; Whitney 1982). In coniferous forests, the most common fungi in beetle galleries are filamentous ascomycetes that are generally known as ophiostomatoids (Harrington 2005; Six 2003). In many countries, ophiostomatoid fungi include species that are involved in tree diseases, cause considerable value loss to the wood product industry, and are considered as quarantine pests (Alfaro *et al.* 2007; Fraedrich *et al.* 2008; Wingfield *et al.* 1993).

The current molecular classification of ophiostomatoids is largely based on nuclear rDNA (rRNA gene). It places the more than 140 species into morphologically similar, non-monophyletic teleomorph genera: *Ceratocystiopsis*, *Ceratocystis*, *Grosmannia* and *Ophiostoma*, as well as into a number of anamorph genera (Hausner *et al.* 2000; Zipfel *et al.* 2006). Species of genus

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Ceratocystis are sensitive to the antibiotic cycloheximide; they are related to the *Microascales*, and are characterised by the *Thielaviopsis* Went anamorph and by phialidic conidia that are produced by the ring wall-building of the collarette (Minter *et al.* 1983). In contrast, species that are tolerant to cycloheximide are characterised by a variety of anamorphs that form conidia by building apical walls (e.g., *Hyalorhinocladiella*, *Leptographium*, *Pesotum* and *Sporothrix*); they are placed into the genera *Ceratocystiopsis*, *Grosmannia* and *Ophiostoma* of the *Ophiostomatales* (Hausner *et al.* 1993; Spatafora & Blackwell 1994; Zipfel *et al.* 2006). *Ceratocystis* species have less specific relationships with their beetle vectors than do members of *Ophiostomatales* teleomorph genera and related anamorphs, which are always associated with scolytid bark beetles (Kirisits 2004).

Ophiostomatoid fungi that were originally called 'ambrosia' are now classified in the anamorph genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* (Batra 1967; Gebhardt *et al.* 2005). 'Ambrosia' represents heterogeneous groups of fungi that include yeasts and filamentous fungi (Batra 1963; Francke-Grosmann 1967). Except for two basidiomycetes (Batra 1972; Hsiau & Harrington 2003), all are ascomycetes, most of which belong to the genera *Ambrosiella* and *Raffaelea*. 'Ambrosia' fungi typically form dense mats of hyphae or clusters of small conidiophores (sporodochia) with conidia that germinate into mass of highly vacuolated sprout cells in beetle galleries; this is often referred to as the 'ambrosia phase' (Batra, 1967). The majority of 'ambrosia' fungi have a symbiotic relationship with platypodid or scolytid ambrosia beetles (Batra 1967; Gebhardt *et al.* 2004, 2005; Harrington *et al.* 2008; Kubono & Ito 2002). These beetles bore galleries into the wood of host trees, and dependent on their fungal symbionts to exploit the nutrient-poor xylem (Batra 1966; Roeper 1995). Many of the beetles have developed mycangia or similar structures that may support transferring fungi to new hosts (Batra 1966; Six 2003). In contrast to ambrosia beetles, scolytid bark beetles feed on the phloem of trees. While bark beetles seem to be less dependent on their fungal associates for nutrition, some may supplement their diets by consuming the fungal associates that they carry on their exoskeletons, and in their guts or mycangia, after the fungi have grown in the beetle galleries (Harrington 2005; Six 2003). *Ambrosiella* species are considered to be typical symbionts of ambrosia beetles; however, a number of *Ambrosiella* fungi are also reported from certain bark beetles (Batra 1967; Kirisits 2004; Krokene & Solheim 1996; Rollins *et al.* 2001). Confusingly, then, the term 'ambrosia' can refer to specific fungal associates and to a particular fungal morphological form (ambrosia phase) in beetle galleries that supports the beetle and its progeny developments (Batra 1967; Hartig 1844).

The taxonomy of 'ambrosia' fungi has been re-evaluated, because their classification was originally established using morphological characteristics that are poorly defined in artificial media, and because most are known only by their asexual state (Batra 1967; Gebhardt *et al.* 2005). For species that lack sexual structures, defining species using only the morphology of asexual phase can be problematic, because many species develop a combination of anamorphs or reduced and non-distinctive asexual structures (Tsuneda & Currah 2006). The genera *Ambrosiella* and *Raffaelea* were differentiated based on the morphology of conidiogenous cells (von Arx & Hennebert 1965); *Raffaelea* have a series of cicatricial conidial scars, while *Ambrosiella* does not. However, applying electron microscopy to a number of *Raffaelea* and *Ambrosiella*, as well as to other asexual ophiostomatoids, has begun to reveal details of conidiogenesis that are not visible by light microscopy (Gebhardt *et al.* 2005; Gebhardt & Oberwinkler 2005).

Molecular phylogenies are clarifying the taxonomic status of most 'ambrosia' fungi among ascomycetes, and specifically in the ophiostomatoids (Cassar & Blackwell 1996; Farrell *et al.* 2001; Gebhardt *et al.* 2005; Jones & Blackwell 1998; Rollins *et al.* 2001). Nuclear small subunit (nSSU) rDNA phylogenies indicate that both *Ambrosiella* and *Raffaelea* are polyphyletic, suggesting that similar morphological characteristics and an intimate association with beetles have originated more than once in these genera (Cassar & Blackwell 1996; Farrell *et al.* 2001). Supporting the initial phylogenies, Gebhardt *et al.* (2005) showed that species of *Ambrosiella* in the order *Microascales* are also morphologically distinct from those in the order *Ophiostomatales*, suggesting that the taxonomic status of genus *Ambrosiella* should be re-evaluated. Despite the close relationship of genera *Ambrosiella* and *Raffaelea* with the ophiostomatoid fungi, they were not included in comprehensive ophiostomatoid fungi phylogenies (Hausner *et al.* 2000; Zipfel *et al.* 2006).

In the work described here, we address where the *Ambrosiella* and *Raffaelea* species should be placed within ophiostomatoids and their relationship to *Ophiostomatales* genera. We report a multigene phylogenetic analysis of filamentous 'ambrosia' fungi that includes twenty-five species from the genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* and thirty species from other ophiostomatoid clades. Our results indicate the limitations of using classical morphological traits and molecular analyses based on single genes to address the taxonomy of these fungi. Finally, we discuss whether the ecological characteristics of the beetle vectors (i.e. bark vs. wood), which originally contributed to the classification of 'ambrosia' fungi, appear to be phylogenetically significant.

Materials and methods

Taxon sampling

Twenty-two strains from the genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* were requested from culture collections (Table 1). We also included three undescribed *Ambrosiella* species (sp. 1, sp. 2 and sp. 3) isolated from spruce-colonising bark beetles in Canada and Europe (Krokene & Solheim 1996; Massoumi Alamouti *et al.* 2007), as well as an undescribed species from the mycangia of ambrosia beetles *Trypodendron rufitarsus* and *Trypodendron lineatum* collected from lodgepole pines infested by the mountain pine beetle (MPB), *Dendroctonus ponderosae* in BC (Kuhnholz 2004). All the fungal strains are maintained at the Breuil Culture Collection (University of British Columbia, BC).

Morphological investigation

Fungal fruiting structures produced from one- to four-week-old cultures grown on Difco malt extract agar (MEA; 20 g Difco malt extract, 10 g Difco agar and 1 L distilled water), potato

Table 1 – Fungal species included in this study

Species	Source[a]	GenBank accession no.[b]		
		nSSU rDNA	nLSU rDNA	β-tubulin gene
Ambrosiella sp. 1	UAMH10632	EU984247	(DQ268582)	(DQ268618)
	UAMH10633	EU984248	(DQ268583)	(DQ268619)
Ambrosiella sp. 2	UAMH10634	EU984249	(DQ268584)	(DQ268620)
	UAMH10635	EU984250	(DQ268585)	(DQ268621)
Ambrosiella sp. 3	NISK-1994-166-39A	**EU984252**	**EU984282**	EU977458
	NISK-1994-176-B4	**EU984253**	**EU984283**	EU977459
A. brunnea	CBS 378.68	(AY858654)	EU984284	EU977460
A. ferruginea	CBS 408.68	**EU984254**	EU984285	EU977461
	JB13[CB]	EU984255	EU984286	EU977462
A. gnathotrichi	CBS 379.68	(AY858655)	EU984287	N/A
A. hartigii	CBS 404.82	**EU984256**	EU984288	EU977463
A. ips	CBS 435.34	AY858657	EU984289	EU977464
A. macrospora	CBS 367.53	**EU984257**	EU984290	EU977465
A. sulcati	CBS 805.70	(AY858658)	EU984291	EU977466
A. sulphurea	CBS 380.68	(AY497509)	EU984292	EU977467
A. tingens	CBS 366.53	**EU984258**	EU984293	EU977468
A. xylebori	CBS 110.61	(AY858659)	EU984294	EU977469
Dryadomyces amasae	CBS 116694	(AY858661)	EU984295	EU977470
Raffaelea albimanens	CBS 271.70	**EU984259**	EU984296	EU977471
R. ambrosiae	CBS 185.64	(AY497518)	EU984297	EU977472
R. arxii	CBS 273.70	(AY497519)	EU984298	N/A
R. canadensis	CBS 168.66	(AY858665)	EU984299	EU977473
R. castellanii	MUCL 15755	EU984260	EU984300	EU977474
R. lauricola		(EU123076)	(EU123077)	N/A
R. montetyi	CBS 451.94	(AY497520)	EU984301	EU977475
R. santoroi	CBS 399.67	**EU984261**	EU984302	EU977476
R. sulcati	CBS 806.70	(AY858666)	N/A	EU977477
R. tritirachium	CBS 726.69	**EU984262**	EU984303	EU977478
Unidentified species	TR25[CB]	EU984251	EU984281	EU977457
Ceratocystiopsis manitobensis	UM 237	EU984266	(DQ268607)	(DQ268638)
Cop. minuta	CBS 463.77	EU984267	(DQ268615)	EU977481
Cop. minuta-bicolor	CBS 635.66	EU984268	(DQ268616)	EU977482
Ceratocystis adiposa	CBS 600.74	EU984263	EU984304	EU977479
C. coerulescens	CL 13-12[CB]	EU984264	(AY214000)	(AY140945)
C. moniliformis	CBS 155.62	EU984265	EU984305	EU977480
Grosmannia abiocarpa	MUCL 18351	EU984269	(AJ538339)	(DQ097857)
G. clavigera	ATCC 18086	EU984270	(AY544613)	(AY263194)
G. cucullata	CBS 218.83	(AY497513)	(AJ538335)	EU977483
G. penicillata		(AY858662)	(DQ097851)	(DQ097861)
G. piceaperda		(AY497514)	(AY707209)	(AY707195)
G. serpens		(AY497516)	(DQ294394)	(AY707188)
Leptographium abietinum	DAOM 60343	EU984271	(DQ097852)	(AY263182)
L. fruticetum	DAOM 234390	EU984272	(DQ097848)	(DQ097855)
L. longiclavatum	DAOM 23419	EU984273	(AY816686)	(AY288934)
L. lundbergii	UAMH 9584	EU984274	(AY544603)	(AY263184)
L. terebrantis	UAMH 9722	EU984275	(AY544606)	(AY263192)
Ophiostoma abietinum	CMW 1468	EU984276	(DQ294356)	EU977484
O. bicolor		(AY497512)	(DQ268604)	(DQ268635)
O. canum	AU 30[CB]	EU984277	(AJ538342)	EU977485
O. floccosum		(AF139810)	(AJ538343)	(AY789142)
O. ips		(AY172021)	(AY172022)	(AY789146)
O. montium	CBS 151.78	EU984278	(AY194947)	(AY194963)
O. novo-ulmi	NAN-MH75[CB]	N/A	(DQ294375)	EU977486
O. piceae		(AB007663)	(AJ538341)	(AY305698)
O. pulvinisporum	CMW 9020	N/A	(DQ294380)	EU977487
O. setosum		N/A	(AF128929)	(AY305703)
O. stenoceras	C80	(M85054)	(DQ294350)	EU977488
O. quercus		(AF234835)	(DQ294376)	(AY789157)
O. ulmi	W9[CB]	(M83261)	(DQ368627)	EU977489
Claviceps sp.		(U32401)	(U17402)	(AF263569)
Daldinia sp.		(U32402)	(U47828)	(AY951701)
Epichloe typhina		(AB105953)	(U17396)	(X52616)

Table 1 – (*continued*)

Species	Source[a]	GenBank accession no.[b]		
		nSSU rDNA	nLSU rDNA	β-tubulin gene
Microascus cirrosus	CBS 217.31	EU984279	(AF275539)	EU977490
Penicillium expansum		(DQ912698)	(AF003359)	(AY674400)
Petriella setifera	CBS 385.87	EU984280	(DQ470969)	EU977491
Taphrina populina		(D14165)	(AF492053)	(AF170968)
Xylaria sp.		(U32417)	(AY327481)	(AY951763)

a Source of isolates sequenced in this study: ATCC, American Type Culture Collection, Manassas, USA; C, Iowa State University, Dept. of Plant Pathology, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW, Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; DAOM, Canadian Collection National Fungus Herbarium and Culture Collection, Ottawa, Canada; NISK, Norwegian Forest Research Institute, Austria; MUCL, (Agro) Industrial Fungi and Yeasts Collection, Belgium; UAMH, University of Alberta Microfungus Collection & Herbarium, Devonian Botanic Garden, Edmonton, Canada; UM, University of Manitoba, Dept. of Botany, Winnipeg, Canada; CB, Colette Breuil's Culture Collection, University of British Columbia, Canada.
b Accession numbers of sequences newly produced, updated (bold) or downloaded from GenBank (parentheses); N/A, not available.

dextrose agar (PDA) and MEA enriched with 1 % Difco yeast extract (YEMEA), were mounted in water and observed using a Zeiss Axioplan compound light microscope. For scanning electron microscopy (SEM), small wood blocks (5 × 2 × 5 mm) bearing fungal structures were fixed using the method described by Lee *et al.* (2003). After fixation, samples were dried with a Blazers CPD 020 critical point drier. They were coated twice with gold palladium using a Nanotech Semprep II sputter coater and examined using a Hitachi S4700 scanning electron microscope.

DNA extraction, PCR amplification and sequencing

DNA was extracted from mycelia grown on Oxoid MEA [33 g malt extract agar (Oxoid CM59), 10 g agar 'tech. No.3' and 1 L distilled water] plates overlaid with cellophane (gel dry grade, BioRad) following the method described by Möller *et al.* (1992). The nSSU was amplified and sequenced with primers NS1 and NS4 (White *et al.* 1990), and the nuclear large subunit (nLSU) region was amplified and sequenced with ITS3 or NL1/LR3 or LROR (O'Donnell 1992; Vilgalys & Hester 1990). The partial β-tubulin gene (β-tubulin) was amplified and sequenced using the primer set BT2E/BT12 (Kim *et al.* 2004). PCR amplification was performed as described by Kim *et al.* (2004). PCR products were purified with a Qiaquick PCR Purification Kit (Qiagen, Ont, Canada). Sequencing was performed on an ABI 3700 automated sequencer (Perkin–Elmer, Foster City, CA) at the DNA synthesis and Sequencing Facility, Macrogen (Seoul, Korea). GenBank accession numbers of the new sequences obtained are shown in Table 1.

Phylogenetic analysis

Sequences from the representatives related to ophiostomatoid fungi in the *Ophiostomatales* and *Microascales*, as well as those representing *Xylariales* and *Hypocreales* were included in the analysis (Table 1). Sequences were aligned using MAFFT (Katoh *et al.* 2002) and then manually adjusted with PHYDIT version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit). The flanking regions were excluded from the analysis because sequence length varied with species. Phylogenetic analysis was conducted for the three loci (nSSU, nLSU and β-tubulin) under both maximum parsimony (MP) methods of PAUP*4.0b10 (Swofford 2003) and Bayesian inference of MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003). Concordance of the three different gene datasets was evaluated with the partition homogeneity test (PHT) implemented with PAUP*4.0b10, using 1000 replicates and the heuristic general search option (Swofford 2003; William & Ballard 1996). *Taphrina populina* and *Penicillium expansum* were assigned as the outgroup taxa (Gebhardt *et al.* 2005; Jacobs *et al.* 2003).

For parsimony analysis, all characters were equally weighted and unordered. Separate analyses were conducted with gaps treated as missing data and as a fifth character state (Swofford 2003). The MP trees (MPTs) were identified by heuristic searches with 100 random stepwise addition replicates and tree-bisection-reconnection branch-swapping algorithms. Statistical support for the branches was assessed by bootstrap analysis (BS) using 1000 MP heuristic searches with ten random sequence addition replicates for each bootstrap replicate. Bayesian inference of phylogeny was calculated based on a Markov chain Monte Carlo analysis with the general time reversible (GTR + I + G) substitution model as determined by AIC criteria of Modeltest (Posada & Crandall 1998). The proportion of alignment sites was assumed to be invariable with gamma-distributed substitution rates of the remaining sites. Four simultaneous Markov chains were run from random starting trees for 1 000 000 generations and sampled every 100 generations (generating 10 001 trees). The first 5000 trees were discarded as burn-in, and inferences of posterior probability (PP) were calculated from 5001 trees.

Results

Sequence analysis

For multigene phylogenetic analysis, we generated fifty-nine rDNA and thirty-five β-tubulin new sequences on the genera *Ambrosiella*, *Raffaelea*, *Dryadomyces*, *Ophiostoma* and *Ceratocystis* and retrieved forty-one sequences of other ophiostomatoid taxa from GenBank (Table 1). We were able to amplify the target loci in all species in the analysis, except for the nLSU locus in *Raffaelea sulcati* and the β-tubulin locus in *Ambrosiella gnathotrichi* and *Raffaelea arxii*. From ophiostomatoid taxa, no

significant length variations were observed in nSSU and nLSU amplicons, whose lengths varied from 831 to 833 and 506 to 539 nucleotides, respectively. However, β-tubulin sequences varied from 550 to 1095 nucleotides. This region contained four exons and 3 introns. Sequences of the four exons were of equal length for all ophiostomatoid taxa in the analysis, whereas sequences of the three introns varied highly in both nucleotide composition and length. Some taxa lacked either one or two introns, which accounted for the large difference in β-tubulin sequence lengths.

The aligned dataset consisted of 837 nucleotides from nSSU, 592 nucleotides from nLSU and 1258 nucleotides from β-tubulin loci. We excluded no nucleotides from nLSU and nSSU loci. However, 735 intron positions were excluded from the β-tubulin locus because the large differences in length and composition of intron sequences across the ophiostomatoid orders and genera made the regions unalignable (Swofford *et al.* 1996).

We submitted the sequences to BLAST to assess potential misidentifications. The comparisons confirmed the species identity of all *Ambrosiella* and *Raffaelea* fungi in the analysis except for *Ambrosiella ips* and *Ambrosiella sulcati*. *A. ips* showed a high level of sequence identity (rDNA + β-tubulin: 99.7 %) with that of *Ophiostoma montium*, suggesting a potential misidentification and the possibility that *A. ips* and *O. montium* might represent a single species. *A. sulcati* showed high sequence identity with that of *Raffaelea canadensis* (rDNA + β-tubulin: 99.6 %), indicating that these two taxa may represent a single species. Since the closest match of *Raffaelea castellanii* was a *Dothideomycetes*, which is unrelated to ophiostomatoid fungi, this species was not included in the final phylogenetic analysis.

Phylogenetic analysis

MPTs from conserved individual loci (nSSU, nLSU and β-tubulin exons) showed weak resolution for the topology of deeper nodes and terminal branches. Although the partition homogeneity test (P-value < 0.01) did not indicate that the rDNA and β-tubulin datasets were concordant, MPT topologies from individual rDNA loci were not in conflict to the combined nSSU + nLSU + β-tubulin dataset, which had better resolution and higher support values. The concatenated matrix (nSSU, nLSU and β-tubulin) included sixty-seven taxa from different ophiostomatoid genera (Fig 1, Table 1) and 1952 aligned sites, of which 719 sites were variable and 534 sites were parsimony informative.

Under the first gap treatment (i.e. gaps as missing data), the parsimony analysis of the concatenated dataset resulted in nine MPTs with a length of 2703 steps (CI = 0.39, RI = 0.71). Gaps as a fifth character state resulted in eleven MPTs with the same length and topologies; thus, for the remainder of the analysis gaps were treated as missing data. The consensus phylogeny inferred from the Bayesian analysis revealed similar topology within and between groupings but with higher supporting relationships than those from the MP analysis (Fig 1). The most visible difference was the placement of the genus *Ceratocystiopsis*, which appeared either as part of the *Ophiostoma* clade or as a basal group to the *Ophiostoma* and *Grosmannia* clades. However, neither method supported the placement of this genus strongly.

Analysis divided the ingroup taxa into four major clades, each receiving 79 % or more bootstrap support and 100 % posterior probabilities (Fig 1A–D). These clades corresponded to the three teleomorph genera *Ophiostoma*, *Grosmannia*, and *Ceratocystiopsis*, recently re-instated by Zipfel *et al.* (2006) in the *Ophiostomatales*, as well as to the genus *Ceratocystis* of the *Microascales*. The twenty-five *Ambrosiella*, *Dryadomyces* and *Raffaelea* species in the analysis were divided into at least six well-resolved (>51 % BS; 100 % PP) groups which nested within the clades of *Ophiostoma* (A), *Grosmannia* (B) and *Ceratocystis* (D) (Fig 1).

Within clade A, *A. ips* grouped strongly (100 % BS) with *O. montium* in the *Ophiostoma ips* complex (group 1) (Zhou *et al.* 2004). Other *Ambrosiella* taxa isolated from scolytid bark beetles formed a single, monophyletic group (group 2) with 99 % BS and 100 % PP (Fig 1). This group contained *Ambrosiella tingens*, *Ambrosiella macrospora* and three undescribed *Ambrosiella* taxa, and was well separated from other *Ambrosiella* species that have been isolated from ambrosia beetles. The bark-beetle associated group showed a sister relationship to a group containing members of the *Ophiostoma piceae* complex (Harrington *et al.* 2001) and both groups are also sibling of the *O. ips* complex (Fig 1A).

Four *Ambrosiella* species clustered with the representatives of *Raffaelea* and *Dryadomyces* in clade B, which also included the genus *Grosmannia*. All 'ambrosia' fungi in this clade had a close association with platypodid and scolytid ambrosia beetles but their monopoly received a poor bootstrap support (62 %) and a low posterior probability of 81 %. Instead they were subdivided into two distinct well-resolved groups (groups 3, 4) that were supported (79 % BS; 100 % PP) as sibling of the genus *Grosmannia*. Group 3, which received strong supports (78 % BS; 100 % PP), encompassed all *Raffaelea* taxa, except *Raffaelea lauricola* and *Raffaelea montetyi*. The group included: *Raffaelea albimanens*, *Raffaelea ambrosiae*, *R. arxii*, *R. canadensis*, *Raffaelea santoroi*, *R. sulcati*, *Raffaelea tritirachium*, as well as two *Ambrosiella* species. *A. sulcati* clustered with *R. canadensis*, with strong support (100 % BS). While *A. gnathotrichi* was closely related to *R. arxii*. These two taxa formed a monophyletic relationship with the *R. canadensis*-clade without bootstrap support and a posterior probability of 95 %.

Representatives of *Raffaelea* concentrated in clade B, but the genus is not monophyletic. However, the relationships among various taxa (*R. albimanens*, *R. santoroi*, *R. sulcati*, *R. tritirachium*) were well resolved. The only exception to this was the unstable positioning of *R. ambrosiae*, the type species of *Raffaelea*, which depending on the locus tested, formed a monophyletic relationship with the *R. arxii* and *R. canadensis* group, or a basal taxon in the group 3.

Group 4, which was supported with high posterior probability (100 %), contained representatives from *Ambrosiella*, *Raffaelea* and *Dryadomyces* but none of these constituted a monophyletic cluster. *Ambrosiella brunnea* and *Ambrosiella sulphurea* mixed with *D. amasea* and with *R. lauricola* and *R. montetyi*. Group 4 also included one unidentified species (TR25) isolated from the mycangia of ambrosia beetle *T. rufitarsus* from lodgepole pines infested by the MPB in BC (Table 2). This fungus appeared as sister of *A. brunnea* and both species formed a well-supported (82 % BS; 100 % PP) monophyletic clade with the recently described species *R. lauricola* (Harrington *et al.* 2008).

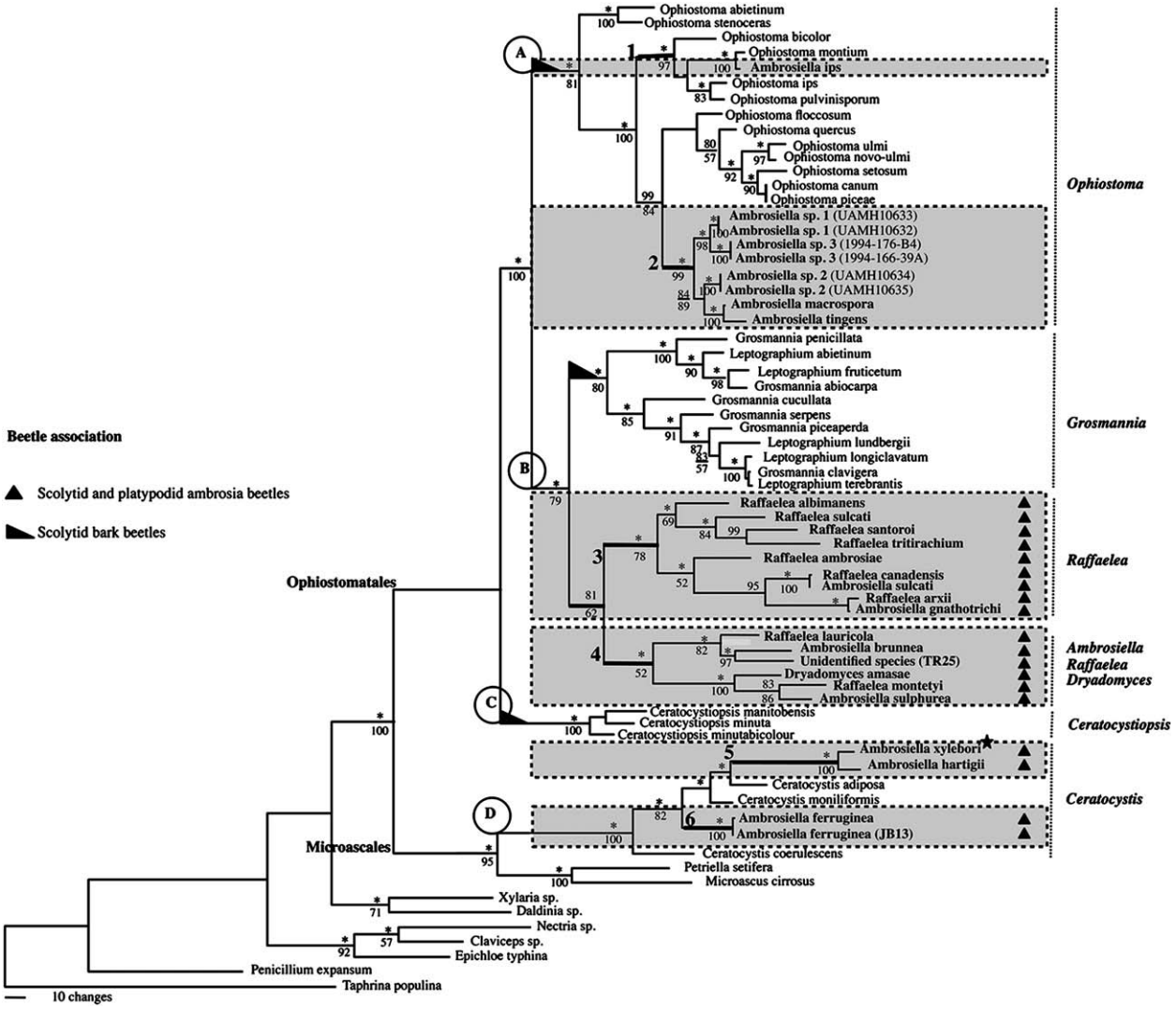

Fig 1 – Phylogenetic relationships of *Ambrosiella*, *Raffaelea* and *Dryadomyces* species with the selected members of different ophiostomatoid teleomorph and anamorph genera. The tree is one of the nine maximum parsimony trees inferred from heuristic analysis of the combined nSSU + 5.8S + nLSU-rDNA + β-tubulin dataset (CI = 0.39, RI = 0.71, length = 2703 steps). Bootstrap percentage values (≥50 %) generated from 1000 replicates from maximum parsimony and posterior probabilities (≥80 %) from Bayesian analysis are shown on the branches. Posterior probabilities of 100 % are shown by *. Thickened black branches represent the major ambrosia clades of genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* produced from the combined dataset. The taxa given in bold represent all the ambrosia fungi included in the analysis. The beetle association (ambrosia and bark beetles) is also mapped onto the phylogenetic tree. *Taphrina populina* and *Penicillium expansum* from orders *Taphrinales* and *Eurotiales*, respectively, were used as outgroup taxa to root the phylogenetic tree.

Three species of *Ambrosiella* belonged to the *Microascales* (clade D) but they did not form a single, monophyletic group (Fig 1). Instead they formed two distinct groups (groups 5, 6) that form a close association with scolytid ambrosia beetles but interspersed with *Ceratocystis* fungi bearing a loose relationship with different insects. First group included the type species *Ambrosiella xylebori* as well as *Ambrosiella hartigii* (group 5) while the other included two strains of *Ambrosiella ferruginea* (group 6).

Morphological investigation

Table 2 summarised morphological features that have been used in the literature to describe the genera *Ambrosiella*, *Raffaelea* and *Dryadomyces*: conidiomatal types (hyphal/singly, sporodochial, and synnematous) and conidial proliferation (annellidic, phialidic, and sympodial).

Representative strains of the *Ophiostoma*-related *Ambrosiella* tended to sporulate better on the PDA and YEMEA media therefore morphological observations were made on these cultures. Similar to other *Ambrosiella*, the undescribed *Ambrosiella* spp. (*Ambrosiella* sp. 1 and *Ambrosiella* sp. 2) isolated from bark beetles in Canada (Massoumi Alamouti *et al.* 2007) and those from European bark beetles (*Ambrosiella* sp. 3) (Krokene & Solheim 1996) produced simple, mononematous conidiophores (Figs 2B and 3); these were arranged in a discrete sporodochium-like structure (Fig 2). Solitary conidiophores were

Table 2 – Morphological and ecological characters reported for the genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* in the literature or by the authors

Fungal species	Morphological characteristic		Ecological characteristic	Reference
	Conidiophore arrangement	Conidiogenesis	Bark beetle ex host tree (geographic origin)	
Ambrosiella sp. 1 (UAMH10632-10633)	Distinct sporodochia-like and solitary[a]	Annellidic & sympodial[a]	*Ips* spp., *Dryocoetes affaber*, *Polygraphus rufipennis* ex *Picea* spp. (Canada)	Beaulieu,[e] Harrison,[e] Massoumi Alamouti *et al.* (2007)
Ambrosiella sp. 2 (UAMH10634-10635)	Distinct sporodochia-like and solitary[a]	Annellidic & sympodial[a]	*Ips* spp., *Dryocoetes affaber*, *Polygraphus rufipennis* ex *Picea* spp. (western Canada)	Massoumi Alamouti *et al.* (2007)
Ambrosiella sp. 3 (NISK-94-166/39A, 1994-176-B4)	Distinct sporodochia-like and solitary (data not shown)[a]	Non-phialidic (data not shown)[a]	*Hylurgops palliatus*, *Polygraphus poligraphus* ex *Picea abies* (Norway)	Krokene & Solheim (1996)
A. brunnea	Distinct sporodochia	Sympodial[b]	*Monarthrum* spp. ex *Acer* spp., *Quercus* spp. (western Canada, USA)	Batra (1967),[d] Funk (1965), Verrall (1943)
A. ferruginea	Effused sporodochia	Phialidic	*Trypodendron* spp., *Xyloterus signatus* ex *Betula* sp., *Fagus sylvatica*, *Larix* spp., *Picea* spp., *Pinus* spp., *Populus* sp., *Quercus* sp. (Canada, Europe, USA)	Batra (1967), Gebhardt *et al.* (2005), Kuhnholz (2004), Mathiesen-Käärik (1953)
[*A. gnathotrichi*][f]	Indistinct sporodochia (fascicles in younger part of colonies)	Non-phialidic	*Gnathotrichus retusus* ex *Picea engelmannii*, *Pinus ponderosa* (Colorado)	Batra (1967), Gebhardt *et al.* (2005)
A. hartigii	Distinct sporodochia	Phialidic	*Xyleborus dispar*, *Xylosandrus germanus* ex *Malus sylvestris* (Asia, Europe, USA)	Batra (1967), Gebhardt *et al.* (2005), Hartig (1844), Kajimura & Hijii (1992)
[*A. ips*]	Solitary (sometimes indistinct sporodochia-like in old cultures)	Sympodial[b]	*Ips* spp., *I. sexdentatus* ex *Pinus* spp. (western USA, Europe)	Batra (1967), Leach *et al.* (1934)
A. macrospora	Effused sporodochia and solitary	Sympodial[b]	*Ips acuminatus* ex *Pinus sylvestris*, *Pinus* spp. (Europe)	Batra (1967), Francke-Grosmann (1952)
[*A. sulcati*]	Distinct sporodochia	Sympodial[b]	*Gnathotrichus retusus* ex *Pseudotsuga menziesii* (Canada)	Funk (1970)
A. sulphurea	Distinct sporodochia	Non-phialidic	*Xyleborinus saxesenii* ex *Populus* spp., *Quercus* spp. (USA, Germany)	Batra (1967), Gebhardt *et al.* (2005)
A. tingens	Solitary or distinct sporodochia	Non-phialidic[a]	*Tomicus minor*, *T. piniperda*, *Ips sexdentatus*, ex *Pinus* spp. (Europe)	Batra (1967), Francke-Grosmann (1952), Mathiesen-Käärik (1953)
A. xylebori	Confluent sporodochia (fascicle in younger part of colonies)	Phialidic	*Xylosandrus compactus*, *X. crassiusculus*, *Corthylus columbianus*, ex *Coffea canephora*, *Acer rubrum* and *Ulmus* sp. (Africa, Ceylon, India, eastern USA, Taiwan)	Batra (1967), Brader (1964), Gebhardt *et al.* (2005), von Arx & Hennebert (1965)
Dryadomyces amasae	Confluent sporodochia	Sympodial	*Amasa* spp. (Taiwan)	Gebhardt *et al.* (2005)
Raffaelea albimanens	Distinct sporodochia, solitary and indeterminate synnemata	Annellidic	*Platypus externedentatus* ex *Ficus sycamorus* (South Africa)	Gebhardt & Oberwinkler (2005), Scott & du Toit (1970),
R. ambrosiae	Distinct sporodochia, solitary or loose fascicles	Annellidic	*Platypus* spp. (i.e. *P. wilsonii*, *P. cylindrus*) ex *Quercus* spp. (British Columbia, England, USA)	Batra (1967), Gebhardt & Oberwinkler (2005), von Arx & Hennebert (1965)
R. arxii	Confluent sporodochia, solitary and indeterminate synnemata	Annellidic	*Xyleborus torquatus* ex *Cussonia umbellif* (South Africa)	Gebhardt & Oberwinkler (2005), Scott & du Toit (1970)
R. canadensis	Solitary and sporodochia	Sympodial[b]	*Platypus wilsonii* ex *Pseudotsuga menziesii* (British Columbia, Oregon)	Batra (1967)

Table 2 – (*continued*)

Fungal species	Morphological characteristic		Ecological characteristic	Reference
	Conidiophore arrangement	Conidiogenesis	Bark beetle ex host tree (geographic origin)	
R. lauricola	Solitary and sporodochia	Sympodial[b]	*Xyleborus glabratus* ex *Persea borbonia*, other members of *Lauraceae* (USA)	Fraedrich *et al.* (2008), Harrington *et al.* (2008)
R. montetyi	Solitary or fascicles in the beetle galleries	Annellidic	*Platypus cylindrus*, *Xyleborus monographus*, *X. dryographusex Quercus* spp. (Europe, Portugal)	Gebhardt *et al.* (2004), Morelet (1998)
R. santori	N/A[c]	Sympodial[b]	*Platypus* spp. (i.e. *P. sulcatus*) ex N/A (Argentine)	Guerrero (1966)
R. sulcati	Effused sporodochia (dense fascicles)	Sympodial[b]	*Gnathotrichus sulcatus* ex *Pseudotsuga menziesii* (Canada)	Funk (1970)
R. quercivora	Distinct sporodochia or solitary	Sympodial[b]	*Platypus quercivorus* ex *Quercus* spp. (Japan)	Kubono & Ito (2002)
R. tritirachium	Confluent sporodochia (fascicles)	Sympodial[b]	*Monarthrum mali* ex *Quercus* spp. (Pennsylvania)	Batra (1967)
Unidentified species (TR25)	N/A	N/A	*Trypodendron rufitarsus* ex *Pinus contorta* (western Canada)	Kuhnholz (2004)

a Reported in this paper.
b Sympodial proliferations have been reported by earlier studies through light microscopy, and therefore the true mode of conidiogenesis in these fungi needs further investigation.
c N/A, no description available.
d References in which the publication year is being shown in parentheses are dealing with the conidiomatal types or conidiogenesis.
e Unpublished data from spruce–beetle–fungal survey in eastern Canada by Dr. Ken Harrison (Natural Resources Canada, Canadian Forest Service-Atlantic) and Marie-Eve Beaulieu in Centre d'étude de la forêt, Université Laval Research.
f Fungi of doubtful genus identity based on the multigene dataset are shown in brackets.

also observed in the younger cultures of these species. Observations by light microscopy of these three species and the *A. tingens* type culture (CBS 366.53) revealed a non-phialidic conidiogenesis (e.g., Fig 3D). In contrast, SEM observations revealed both annellidic (Figs 2B and 3A,B) and sympodial (Fig 3C) conidiogenesis in *Ambrosiella* sp. 1 as well as in *Ambrosiella* sp. 2; however, conidial development seemed to occur more frequently through annellidic percurrent proliferation than sympodial proliferation. Because *A. macrospora*, *A. tingens* and the species from Europe produced few spores on artificial media and wood blocks, we were unable to determine with certainty whether their conidiogenesis was sympodial or annellidic. *A. ips* (CBS 435.34) also failed to produce any fruiting structures on media and therefore its morphological characters could not be compared to those of the genetically identical species *O. montium* (CBS 151.78).

Discussion

We established a comprehensive phylogeny that clarifies the relationships between most filamentous 'ambrosia' fungi isolated from platypodid and scolytid beetles and their relationships with the ophiostomatoid fungi. Our results are consistent with studies that described the polyphyletic status of the genera *Ambrosiella* and *Raffaelea* (Cassar & Blackwell 1996; Farrell *et al.* 2001; Gebhardt *et al.* 2005; Rollins *et al.* 2001). These earlier phylogenies used mainly nSSU rDNA sequences to characterise members of *Ambrosiella* and/or *Raffaelea* at higher taxonomic levels, and sets of species that that did not adequately represent the morphological and ecological diversity of ophiostomatoid fungi. In the work described here, we addressed both limitations. We generated a new multigene dataset, and we characterised a diverse set of fungi that included representatives from the genera *Ambrosiella*, *Raffaelea* and *Dryadomyces*, as well as from taxa that we selected from currently accepted ophiostomatoid teleomorph and anamorph genera. Our analysis indicated that these fungi evolved from three major teleomorph groups in two ascomycete orders: *Ophiostomatales* and *Microascales*. We will not discuss the *Ambrosiella* species within *Microascales* that include the type species *A. xylebori* because this aspect has been thoroughly studied by Paulin-Mahady *et al.* (2002) and their results agree with ours. In the subsequent discussion we will focus on the *Ambrosiella* species that belong to the *Ophiostomatales*.

von Arx & Hennebert (1965) introduced the genus *Ambrosiella* to describe *A. xylebori*, the most frequent associate of the ambrosia beetle *Xylosandrus compactus*. Following the original description of the type species, nine additional species were assigned to the genus (Batra, 1967). These species share certain morphological features: simple conidiophores, sporodochial arrangements of conidiophores and single terminal conidia (Batra 1967). Cassar & Blackwell (1996) showed that the genus *Ambrosiella* was not monophyletic within

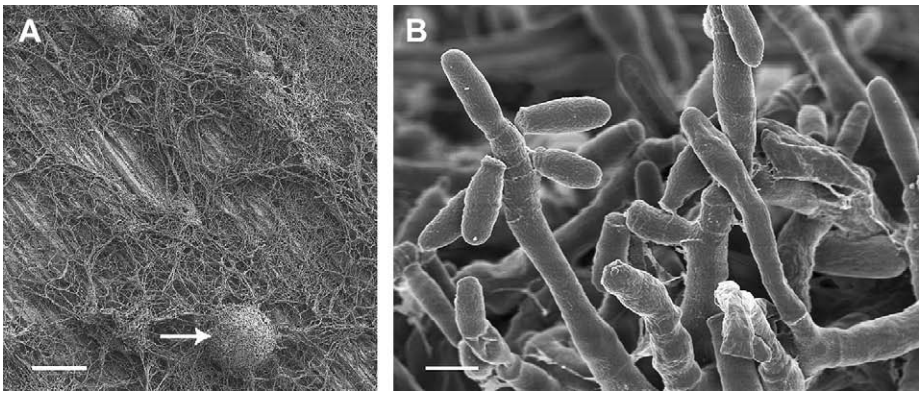

Fig 2 – Scanning electron micrographs of sporodochium-like structures developed by the *Ophiostoma*-related *Ambrosiella* (*Ambrosiella* sp. 2 UAMH 10635). (A) Low magnification of sporodochium-like structures developed from an interwoven mat of hyphae. (B) Close-up of the conidiophores and annellidic conidiogenous cells comprising a portion of the sporodochium-like structures indicated in Fig 2A (arrow). Bar = (A) 300 μm, (B) 2.5 μm.

Ophiostomatales based on nSSU rDNA. Their phylogenies recognised two possible *Ambrosiella* groups that were closely related to either *Leptographium*-forming species or *Ophiostoma* species characterised by their *Pesotum* (e.g., *O. piceae*) and/or *Hyalorhinocladiella* (e.g., *O. bicolor*) anamorphs. Results from Farrell *et al.* (2001), Rollins *et al.* (2001) and Gebhardt *et al.* (2005) supported these groupings, but resolved neither the monophyly of different *Ambrosiella* groups nor their relationships with the closely related genera *Ophiostoma* and *Raffaelea*. Our multigene phylogeny clarified the relationships among these fungi and recognised at least four groups of *Ambrosiella* within the *Ophiostomatales*.

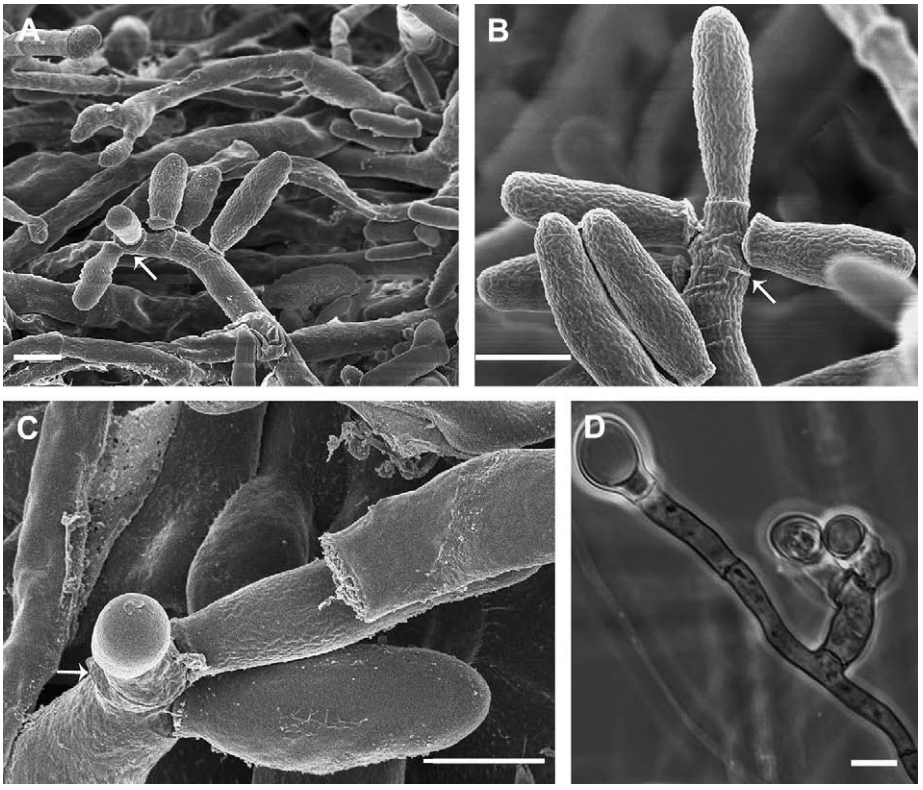

Fig 3 – Scanning electron micrographs (A–C) and light micrograph (D) of the anamorphic structure of *Ophiostoma*-related *Ambrosiella*. (A, B) Annellidic conidiogenous cells (arrowed) of *Ambrosiella* sp. 1 UAMH 10632 and *Ambrosiella* sp. 2 UAMH 10635, respectively, arising from mononematous conidiophores tapering toward the apex. (C) Conidiogenous cell of *Ambrosiella* sp. 2 UAMH 10635 showing apical conidia produced through sympodial proliferation (arrowed). (D) *A. tingens* CBS 366.53, producing conidia through the non-phialidic conidiogenesis. Bars = 2.5 μm.

Ambrosiella *associates of bark beetles are related to the teleomorph genus* Ophiostoma

Our results supported a novel clade that consisted of five bark-beetle associates: *A. macrospora*, *A. tingens* and the three undescribed *Ambrosiella* species from Canadian and European bark beetles (Krokene & Solheim 1996; Massoumi Alamouti *et al.* 2007). *A. macrospora* and *A. tingens* were originally described in the genus *Trichosporium* as *Trichosporium tingens* var. *macrosporum* and *Trichosporium tingens* (Francke-Grosmann 1952; Lagerberg *et al.* 1927). Batra (1967) reclassified these two bark-beetle associates into *Ambrosiella*, while indicating that this genus should describe fungal associates of platypodid and scolytid ambrosia beetles. nSSU rDNA phylogenies subsequently showed that these two species were more closely related to *Ophiostoma* than to *Ambrosiella* (Cassar & Blackwell 1996; Rollins *et al.* 2001); however, limitations from nSSU rDNA sequences and taxon sampling prevented these studies from characterising the phylogenetic relationships in detail. In contrast, our multigene analysis resolved the *Ambrosiella* associates of bark beetles as a distinct group within *Ophiostoma*. We also showed that the bark-beetle associated group is a sibling to the *O. piceae* complex, members of which commonly inhabit sapwood and bark-beetle tunnels in temperate forests (Harrington *et al.* 2001). Members of this complex are distinguished by their teleomorph fruiting bodies and *Pesotum* anamorph, which include both synnematous and *Sporothrix* conidiophore arrangements (Harrington *et al.* 2001; Seifert & Okada 1993). *Ambrosiella* species produce conidiophores without denticles (Batra 1967; Gebhardt *et al.* 2005), which differentiate them from *Sporothrix*. *Ambrosiella* associates of bark beetles, including the undescribed species, do not produce synnemata; instead, their conidiophores are arranged in distinct sporodochial-like structures that resemble those of other *Ambrosiella* and *Raffaelea* species. Our analysis also strongly suggested that the bark beetle-associated *Ambrosiella* and the *O. piceae* complex form a monophyletic clade with members of the *O. ips* complex, which are distinguished by their pillow-shaped ascospores and continuum of anamorphs including *Hyalorhinocladiella* and *Pesotum* (Zipfel *et al.* 2006). This monophyletic clade is a sister to another *Ophiostoma* group that includes a number of species (e.g., *Ophiostoma abietinum* and *Ophiostoma stenoceras*) found in a diverse range of ecological niches and identified by their *Sporothrix* anamorph.

Our analysis grouped *A. ips* with *O. montium* within the *O. ips* complex. Originally *A. ips* was described in the *Tuberculariella* (Leach *et al.* 1934), and then was transferred into the genus *Ambrosiella* (Batra, 1967). However, our molecular results, as well as morphological and ecological descriptions from the literature suggested that these two species might represent a single taxon. We showed that the two species shared a high level of sequence identity (99.7 %). We were unable to compare the morphology of *A. ips* and *O. montium* because, in our hands, the only *A. ips* strain available from the CBS culture collection and reported in the literature did not sporulate; however, the sporodochium-like structures illustrated in the description of *A. ips* (Leach *et al.* 1934) are similar to *Graphilbum* reported for *O. montium* and *O. ips* (Hutchison & Reid 1988; Upadhyay 1981). Because both *A. ips* and *O. montium* have been isolated from bark beetles (*Ips. pini* and MPB, respectively) that infest the same pine-host trees in North America (Leach *et al.* 1934; Lee *et al.* 2006), it is possible that galleries of these beetles have overlapped, resulting in fungal associates being mixed and *A. ips* being misidentified.

Ambrosiella *associates of ambrosia beetles are related to the teleomorph genus* Grosmannia

In our analysis, the remaining *Ophiostomatales Ambrosiella* grouped with members of genera *Raffaelea* and *Dryadomyces* and formed a sister relationship with members of the genus *Grosmannia*. All members of this group (*Raffaelea*, *Dryadomyces* and *Ambrosiella*) are closely associated with the platypodid and scolytid ambrosia beetles (Batra 1967; Funk 1970; Guerrero 1966; Scott & du Toit 1970; von Arx & Hennebert 1965). Our multigene phylogenies suggested that these ambrosia-beetle associates are monophyletic but with weak statistical support. Note that while we included most species described from ambrosia beetles, relatively few such associates have been fully characterised, and we were unable to further test the monophyly with sequence data for more isolates. The placement of ambrosia-beetle associates within the *Ophiostomatales* has been problematic because earlier phylogenetic studies consistently grouped them with species like *Ophiostoma piceaperdum* and *Ophiostoma serpens* of the well-defined genus *Grosmannia* (Gebhardt *et al.* 2005; Hulcr *et al.* 2007; Rollins *et al.* 2001). Zipfel *et al.* (2006) re-instated this genus to accommodate the most common fungal associates of bark beetles that are distinguished by their *Leptographium* anamorph (Jacobs & Wingfield 2001); however, when they tested the monophyly of *Grosmannia* they did not consider the close relatives of this genus, namely, members of genera *Ambrosiella* and *Raffaelea*. While our results confirmed that *Grosmannia* is monophyletic, we also included the *Grosmannia*-related associates of ambrosia beetles. Our analysis placed these into two distinct groups and provided the first robust indication that the ambrosia-beetle associates are close but independent relatives of *Leptographium*-forming species commonly isolated from bark beetles.

The first of the two groups included seven of the nine tested species of genus *Raffaelea*, as well as *A. sulcati* and *A. gnathotrichi*. The exceptions, *R. lauricola* and *R. montetyi*, were placed in the second group. The *Raffaelea* genus was established by von Arx & Hennebert (1965) to describe *R. ambrosiae*, which is frequently associated with the pinhole borer *Platypus cylindricus* in North America and Europe. Because the group members have similar ecological and morphological features and formed a strongly supported monophyletic clade, we suggested that they should be all recognised as species of genus *Raffaelea s. str.* (Batra 1967; Funk 1970; Guerrero 1966; Scott & du Toit 1970; von Arx & Hennebert 1965). We summarised evidence for this as follows. *A. sulcati* and *R. canadensis* were respectively isolated from the ambrosia beetles *Gnathotrichus retusus* and *Platypus wilsonii* when these two beetles inhabited the same host, *Pseudotsuga menziesii* (Douglas fir) (Batra 1967; Funk 1970). Differentiating *Raffaelea* and *Ambrosiella* morphologically by assessing whether cicatricial conidial scars are present or absent using light microscopy is difficult, and often

depends on subtle interpretations by researchers (Batra 1967; Funk 1970; von Arx & Hennebert 1965). However, our results showed that these two species have high rDNA and β-tubulin sequence identify (99.6 %) and formed a conspecific group within *Raffaelea* species, and so indicated that *R. canadensis* and *A. sulcati* represent a single taxon and that *A. sulcati* should be transferred into *Raffaelea*. *A. gnathotrichi* is a frequent associate of the conifer-infesting species *G. retusus* in North America (Batra, 1967). While *R. arxii* forms a close association with the *Xyleborus torquatus* on the *Cussonia umbellif* in South Africa (Scott & du Toit 1970). Gebhardt *et al.* (2005) suggested that *A. gnathotrichi*'s conidial ontogeny differs from that of the *Ambrosiella* type species and they showed that *A. gnathotrichi* form a sister taxon relationship with *R. arxii*. Our multigene analysis provided a higher resolution for the species-level phylogeny and showed that these two fungi are closely related species. Although, our analyses suggest that *A. gnathotrichi* be assigned to the genus *Raffaelea*, a more thorough morphological examination of the type material is needed, particularly of conidial ontogeny.

The second group included the remaining species from all three genera: *Ambrosiella*, *Raffaelea* and *Dryadomyces*. The group members were *A. brunnea*, *A. sulphurea*, *R. lauricola*, *R. montetyi* and *Dryadomyces amasae*, and an unidentified species isolated from *T. rufitarsus* colonising MPB-attacked lodgepole pine in BC (Kuhnholz 2004). Our phylogenetic analysis consistently resolved these species as sister of other ambrosia-beetle associates in the *Raffaelea* clade, with weak statistical support. We will clarify briefly the group members. *A. brunnea* and *A. sulphurea* have been isolated from North American and European hardwood species (*Quercus* and *Acer*) that were infested with ambrosia beetles in the genus *Monarthrum* and with *Xyleborus saxesenii*, respectively (Batra 1967; Verrall 1943). Gebhardt *et al.* (2005) showed that these two *Ambrosiella* species produce a non-phialidic conidiogenesis, and included them in their *Raffaelea* phylogenetic clade. In contrast, our analysis segregated them from both *Ambrosiella* and *Raffaelea* genera. Our analysis showed that the *Ambrosiella* associate of *Monarthrum* spp. (*A. brunnea*), and the unidentified species isolated from *T. rufitarsus* in BC are sister taxa that form a distinct, well-supported monophyletic group with the new vascular wilt pathogen *R. lauricola* (Fraedrich *et al.* 2008). This pathogen is associated with the exotic ambrosia beetle *Xyleborus glabratus*, and causes substantial mortality of redbay and other *Lauraceae* in the USA (Fraedrich *et al.* 2008; Harrington *et al.* 2008). nSSU and nLSU sequences from Fraedrich *et al.* (2008) respectively suggested that the pathogen is related to *A. brunnea* and *Leptographium* spp. Our phylogenetic results were consistent with this pathogen being a distinct species; and included it with other ambrosia-beetle associates of genera *Ambrosiella*, *Raffaelea* and *Dryadomyces* that formed a sister group relationship with *Leptographium*-forming species. Harrington *et al.* (2008) suggested that this fungus most appropriately fits the genus *Raffaelea*; however, they did not provide a detailed morphology of the conidiogenous cells. Further, our analyses placed this pathogen in a clade that was clearly separated from other ambrosia-beetle associates in the genus *Raffaelea*. Therefore, the taxonomic status of the vascular wilt pathogen in the USA is not clear and needs further study. Our multigene phylogenies placed *A. sulphurea* into a distinct subclade that formed a highly supported sister relationship with *R. montetyi*. Previous nSSU phylogenies had also suggested that these two species were closely related (Gebhardt *et al.* 2004, 2005; Hulcr *et al.* 2007). Both *A. sulphurea* and *R. montetyi* are frequently associated with ambrosia beetles in the genus *Xyleborus* that inhabit the oak trees (Gebhardt *et al.* 2004). Although, *R. montetyi* has been shown to produce conidia by annellidic percurrent proliferation, resolving the taxonomic placement of *R. montetyi* and that of *A. sulphurea* will require additional morphological studies. Finally, the second group comprises a monotypic genus, *Dryadomyces*, which accommodates the single species *D. amasae*. The genus was introduced by Gebhardt *et al.* (2005) to describe fungi frequently isolated from ambrosia beetle *Amasa concitatus* infesting hardwood timbers in Taiwan. nSSU results indicated that these fungi were phylogenetically related to species of genera *Raffaelea* and *Ambrosiella* but they were included into the new genus *Dryadomyces* based on their unique conidiogenous cells (Gebhardt *et al.* 2005). Later, Harrington *et al.* (2008) amended the genus indicating that until the taxonomy of the genus *Ophiostoma* was better resolved, *Raffaelea* should include all ambrosia-beetle symbionts with affinities to *Ophiostoma*. Our multigene analysis provided a better resolution for the phylogenetic status of the genus *Ophiostoma* and consistent with the morphological observations of Gebhardt *et al.* (2005), recognised *D. amasae* as a distinct monotypic lineage that formed a highly supported monophyletic relationship with *R. montetyi* and *A. sulphurea*.

Morphological features

Morphological characters used to define *Ambrosiella* are less informative in phylogeny because the genus is polyphyletic. The morpho-taxonomy of *Ambrosiella* and *Raffaelea* has been difficult and unstable. The shape (reduced conidiophores) and arrangement of conidiophores (sporodochia), as well as the mode of conidiogenesis are the key morphological characteristics traditionally used to differentiate the genus *Ambrosiella* from the closely related genus *Raffaelea* (Batra, 1967; von Arx & Hennebert 1965). However, complex or simple conidiophores did not correlate with the generic or sub-generic classification. Also the mode of conidiogenesis is difficult to observe under light microscopy (Tsuneda & Currah 2006). *Ambrosiella* and *Raffaelea* genera were reported as sympodial (von Arx & Hennebert 1965). But, Gebhardt *et al.* (2005) showed the presence of phialidic conidia for *A. xylebori*, and in other two *Ceratocystis*-related species: *A. hartigii* and *A. ferruginea*. Recently *Raffaelea* species having annellidic conidiogenesis and sympodial proliferations in *D. amasae* were also illustrated (Gebhardt *et al.* 2004; Gebhardt & Oberwinkler 2005).

Our phylogenetic results also indicated that the characters used to define anamorphs are convergent within the *Ophiostomatales*. The conidial proliferation in the unidentified *Ambrosiella* species, as well as *A. tingens* was non-phialidic, which clearly distinguished them from the type species of *Ambrosiella*. In addition, we observed that the conidiophores of these strains lacked denticles; this differentiated them from anamorph genera *Sporothrix* and *Dryadomyces*, both of which have conidia formed sympodially on denticles arising from undifferentiated hyphae (Gebhardt *et al.* 2005; Harrington

et al. 2001). In our SEM micrographs, we found that the non-phialidic conidiogenesis observed for *Ambrosiella* sp. 1 (Fig 3A) and *Ambrosiella* sp. 2 (Fig 3B) was occurring through annellidic proliferation, and consequently was identical to that found for *Ophiostomatales* anamorph genera *Raffaelea*, *Hyalorhinocladiella*, *Leptographium* and *Pesotum* (Benade *et al.* 1995; Seifert & Okada 1993; Tsuneda & Currah 2006). These two undescribed *Ambrosiella* also formed apical sympodial conidiogenesis but less frequently. Although, additional work is necessary to define the true mode of conidiogenesis for all *Ambrosiella* species related to the *Ophiostoma*, it is important to note that the conidium ontogeny, an early important taxonomic character, is now being challenged, because conidial fungi often develop more than one pattern of conidiogenesis and can be assigned to different anamorphic genera (Tsuneda & Currah 2006).

While *Ambrosiella* sp. 1 and *Ambrosiella* sp. 2 with their annellidic conidiogenesis and the morphology of their conidiophores are most similar to species of the genus *Raffaelea*, they were clearly distinguished from the *Raffaelea* group by our multigene phylogeny. Consistent with our phylogenetic classification, members of *Raffaelea* clade also colonise different ecological niches. The morphological characteristics of *Ambrosiella* sp. 1 and *Ambrosiella* sp. 2 also resemble those of *Hyalorhinocladiella* anamorph. This anamorphic state is not clearly delimited to a genus and is present in anamorphs of *Ceratocystiopsis* and *Ophiostoma* (e.g., *O. ips* complex) (Benade *et al.* 1996; Upadhyay & Kendrick 1975). Currently, species of genera *Ambrosiella* and *Raffaelea* are differentiated from other ophiostomatoid genera including *Hyalorhinocladiella* by the formation of sporodochia. However, the production of sporodochia is variable and is often associated with the growth of the fungus in its natural habitat (beetle gallery); as well, the importance of this structure for segregating anamorphic genera within the ophiostomatoid fungi has not been clarified.

Conclusions

We clarified the phylogenetic classification of *Ambrosiella* species isolated from ambrosia and bark beetles and that of the *Raffaelea* and *Dryadomyces* associates of ambrosia beetles, as well as the relationships between these species and ophiostomatoid relatives. We found that species of genus *Ambrosiella* are distributed in a number of distinct phylogenetic groups that each might be reassigned to different genera, and that the genus *Raffaelea* should be revised. While no morphological characteristics unambiguously supported the monophyletic groups that we report from our molecular data for the genera *Ambrosiella* and *Raffaelea*, these groups are clearly associated with the feeding behaviour of their beetle vectors. Specifically, *Ambrosiella* associates of scolytid bark beetles formed a monophyletic group in the genus *Ophiostoma*, while species associated with scolytid and platypodid ambrosia beetles form separate lineages that have a monophyletic relationship with the genus *Grosmannia*. Generating additional support for the monophyly presented will require characterising a range of morphological characters and/or ecological traits on an expanded collection of freshly isolated fungi from ambrosia and bark beetles.

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