British Mycological Society promoting fungal science

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

Phylogeny of ambrosia beetle symbionts in the genus *Raffaelea*

Tyler J. DREADEN[a], *John M. DAVIS*[a], *Z. Wilhelm DE BEER*[b], *Randy C. PLOETZ*[c], *Pamela S. SOLTIS*[d], *Michael J. WINGFIELD*[b], *Jason A. SMITH*[a,*]

[a]*School of Forest Resources and Conservation, University of Florida, Gainesville 32611, USA*
[b]*Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa*
[c]*Tropical Research and Education Center, University of Florida, Homestead 33031, USA*
[d]*Florida Museum of Natural History, University of Florida, Gainesville 32611, USA*

ARTICLE INFO

Article history:
Received 16 July 2014
Received in revised form
11 September 2014
Accepted 15 September 2014
Available online 22 September 2014

Keywords:
Internal transcribed spacer region
ITS
Ophiostomatales
Raffaelea sensu stricto
Wilt pathogens

ABSTRACT

The genus *Raffaelea* was established in 1965 when the type species, *Raffaelea ambrosia*, a symbiont of *Platypus* ambrosia beetles was described. Since then, many additional ambrosia beetle symbionts have been added to the genus, including the important tree pathogens *Raffaelea quercivora*, *Raffaelea quercus-mongolicae*, and *Raffaelea lauricola*, causal agents of Japanese and Korean oak wilt and laurel wilt, respectively. The discovery of new and the dispersal of described species of *Raffaelea* to new areas, where they can become invasive, presents challenges for diagnosticians as well as plant protection and quarantine efforts. In this paper, we present the first comprehensive multigene phylogenetic analysis of *Raffaelea*. As it is currently defined, the genus was found to not be monophyletic. On the basis of this work, *Raffaelea sensu stricto* is defined and the affinities of undescribed isolates are considered.

Introduction

Both *Raffaelea* and *Ambrosiella* species colonize the natal galleries of ambrosia beetles in tree sapwood, and they maintain close associations with these insects (Batra 1967). Although most *Raffaelea* spp. live as saprophytes, colonizing dead and dying wood, some species such as *Raffaelea lauricola*, *Raffaelea quercivora*, and *Raffaelea quercus-mongolicae* are serious pathogens that can cause significant damage to forests and fruit crops (Kim *et al.* 2009; Kubono & Ito 2002; Ploetz *et al.* 2013). The causal agent of laurel wilt, *R. lauricola*, is highly virulent and able to cause systemic wilt from a single inoculation. It threatens native Lauraceae in the southeastern United States and avocado production in Florida (Ploetz *et al.* 2011, Ploetz *et al.* 2013). Thus, the discovery of new taxa and the dispersal of known taxa to new areas may represent important threats to forests and agriculture. Clarification of the taxonomy of *Raffaelea*, and related genera, would clearly aid researchers and

* *Corresponding author*. School of Forest Resources and Conservation, PO Box 110410, University of Florida, Gainesville, FL 32611-0410, USA. Tel.: +1 352 846 0843.
E-mail address: jasons@ufl.edu (J. A. Smith).
http://dx.doi.org/10.1016/j.funbio.2014.09.001
1878-6146/

diagnosticians who deal with these important challenges. Additionally, clear taxonomy and a strong phylogeny of the genus would allow for an examination of the evolutionary biology of the ambrosial symbioses.

The genus *Raffaelea* was established by Arx & Hennebert (1965) to accommodate *Raffaelea ambrosiae*, a symbiont of *Platypus* ambrosia beetles; it currently includes up to 20 described species (Harrington *et al.* 2010; De Beer *et al.* 2013b). *Raffaelea* has traditionally been distinguished from *Ambrosiella* by the sympodial proliferation of the conidiogenous cells in *Raffaelea* and percurrent proliferation of the conidiogenous cells in *Ambrosiella* (Batra 1967; Harrington *et al.* 2008). This distinction is difficult to discern microscopically, and its utility to distinguish the two genera has been questioned (Gebhardt & Oberwinkler 2005; Harrington *et al.* 2008). Molecular phylogenetic approaches have been used to clarify the taxonomic relationships of most groups of fungi, including the Ophiostomatales (Duong *et al.* 2012; Farrell *et al.* 2001; James *et al.* 2006; Slippers *et al.* 2013; Wingfield *et al.* 2013). Ribosomal DNA sequence data have confirmed that the two genera are not closely related, as *Raffaelea* resides in the Ophiostomatales and *Ambrosiella* in the Microascales (Cassar & Blackwell 1996; Jones & Blackwell 1998; De Beer *et al.* 2013a).

The relationships between *Raffaelea* and related genera and their placement within the Ophiostomatales have not been fully resolved. The genus name *Dryadomyces* was introduced by Gebhardt *et al.* (2005) to accommodate *Dryadomyces amasae* (=*Raffaelea amasae*). It fell in the *Raffaelea* clade in their phylogenetic analyses of the rDNA small ribosomal subunit (SSU) sequences, but based on conidiogenesis, it differed from *Raffaelea*. Harrington *et al.* (2008) reduced *Dryadomyces* to synonymy with *Raffaelea*, supporting the view that all ambrosia beetle symbionts with similarities to *Ophiostoma* should be included in *Raffaelea*. Massoumi Alamouti *et al.* (2009) conducted a multigene phylogenetic analysis of a limited sampling of ambrosia fungi. They showed that *D. amasae* grouped in a monophyletic lineage distinct from the lineage containing *R. ambrosiae*, the type species for *Raffaelea*. However, Harrington *et al.* (2010) revised *Raffaelea* and maintained the synonymy of *Dryadomyces* with *Raffaelea*. In a taxonomic review of the Ophiostomatales, De Beer & Wingfield (2013) contextualized the phylogenetic placement of *Raffaelea* spp. alongside all other accepted genera within the order based on available rDNA large ribosomal subunit (LSU) data, confirming the polyphyly of the genus as suggested by Massoumi Alamouti *et al.* (2009). They defined *Raffaelea sensu stricto*, as well as two distinct clades. In one clade, *R. lauricola*, *Raffaelea brunnea*, and an undescribed species from Canada were included in *Ophiostoma sensu lato*, but the definition of what should be included in *Ophiostoma* was vague. The second clade included *R. quercivora*, *Raffaelea montetyi*, *Raffaelea sulphurea*, and *R. amasae* in *Leptographium sensu lato* (De Beer & Wingfield 2013). These authors concluded that additional data would be required to fully resolve the generic status of these two unrelated clades accommodating diverse species of *Raffaelea*.

The objectives of this study were to conduct multigene phylogenetic analyses of *Raffaelea* spp. and to test the monophyly of the genus as it is currently defined. An additional objective was to assess the affinity of a collection of isolates that have yet to be identified.

Materials and methods

Taxon sampling

Data from previous studies were assessed and the LSU, SSU, and β-tubulin (BT) loci were selected for the present study because they have been useful for constructing phylogenies for these fungi and are available in GenBank (Massoumi Alamouti *et al.* 2009; Harrington *et al.* 2010; De Beer & Wingfield 2013). In all, 77 isolates were analysed, including nine in the Microascales and 55 in the Ophiostomatales (18 species of *Ophiostoma*, three of *Ceratocystiopsis*, 11 of *Grosmannia*, one of *Esteya*, two of *Fragosphaeria*, and all 20 species of *Raffaelea* that were defined by Harrington *et al.* (2010)) (Table 1). Unidentified isolates and outgroup taxa comprised the remaining isolates. Sequences were either acquired from GenBank or obtained by sequencing (Table 1).

DNA extraction, PCR amplification, and sequencing

Polymerase chain reactions were performed using DNA that was extracted from cultures (Justesen *et al.* 2002; Duong *et al.* 2012) using PCR primer pairs NL1/LR3, NS1/NS4, and Bt2a/Bt2b for the LSU, SSU, and BT loci, respectively (Glass & Donaldson 1995; O'Donnell 1993; Vilgalys & Hester 1990; White *et al.* 1990). Sanger sequencing was performed using the same primers at the University of Florida Interdisciplinary Center for Biotechnology Research, and consensus sequences were constructed using both the forward and reverse sequence reads using Geneious Pro 5.6.6 (Biomatters, Auckland, New Zealand). After many attempts, rDNA internal transcribed spacer region ITS1-5.8s-ITS2 (ITS) PCR amplicons were generated for several *Raffaelea* spp. isolates using FastStart *Taq* with the GC-RICH solution (Roche Applied Science, Basel, Switzerland) and primers ITS1F/ITS4 (Gardes & Bruns 1993; White *et al.* 1990). Sanger sequencing of ITS amplicons was performed at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and aligned as above.

Phylogenetic analyses

DNA sequences were aligned with sequences retrieved from GenBank (Table 1) using the Geneious alignment default settings in Geneious Pro 5.6.6, manually adjusted, and then trimmed. The introns in the BT loci could not be unambiguously aligned and were removed from the dataset. The presence or absence of the BT introns was also coded, but gave maximum parsimony (MP) results similar to the non-intron-coded dataset and was not used in subsequent analyses. Congruence among the three datasets was first evaluated using the partition-homogeneity test (PHT) in PAUP* 4.0a129, with a heuristic search, tree-bisection-reconnection (TBR) branch swapping algorithm and Maxtree set to auto increase, and again using Maxtree = 500 with both TBR and nearest-neighbor interchange (NNI) branch swapping algorithms (Swofford 2003). Congruence among gene trees was evaluated by conducting a maximum likelihood (ML) analysis on each gene (Fig S2–S4), and then comparing the results visually.

Table 1 – Taxon names, isolate, and GenBank accession numbers used in the study.

Taxon isolate	Accession		
	LSU	SSU	BT
Ambrosiella ferruginea CBS408.68	EU984285	EU984254	EU977461
Ambrosiella ferruginea JB13	EU984286	EU984255	EU977462
Ambrosiella hartigii CBS404.82	EU984288	EU984256	EU977463
Ambrosiella xylebori CBS110.61	EU984294	AY858659	EU977469
Ceratocystiopsis manitobensis UM237	DQ268607	EU984266	DQ268638
Ceratocystiopsis minuta CBS463.77	DQ268615	EU984267	EU977481
Ceratocystiopsis minuta-bicolor CBS635.66	DQ268616	EU984268	EU977482
Ceratocystis adiposa CBS600.74	EU984304	EU984263	EU977479
Ceratocystis coerulescens CL13-12	AY214000	EU984264	AY140945
Ceratocystis moniliformis CBS155.62	EU984305	EU984265	EU977480
Claviceps fusiformis ATCC26019	U17402	DQ522539	AF263569
Daldinia concentrica	U47828	U32402	FJ185285
Epichloe typhina	U17396	AB105953	X52616
Esteya vermicola CBS115803	EU668903		FJ490552
Fragosphaeria purpurea CBS133.34	AF096191	AF096176	
Fragosphaeria reniformis CBS134.34	AB189155	AB278193	
Grosmannia abiocarpa MUCL18351	AJ538339	EU984269	DQ097857
Grosmannia clavigera ATCC18086	AY544613	EU984270	AY263194
Grosmannia cucullata	AJ538335	AY497513	EU977483
Grosmannia penicillata	DQ097851	AY858662	DQ097861
Grosmannia piceiperda	AY707209	AY497514	AY707195
Grosmannia serpens	DQ294394	AY497516	AY707188
Leptographium abietinum DAOM60343	DQ097852	EU984271	AY263182
Leptographium fruticetum DAOM234390	DQ097848	EU984272	DQ097855
Leptographium longiclavatum	AY816686	EU984273	AY288934
Leptographium lundbergii UAMH9584	AY544603	EU984274	AY263184
Leptographium terebrantis UAMH9722	AY544606	EU984275	AY263192
Microascus cirrosus CBS217.31	AF275539	EU984279	EU977490
Ophiostoma abietinum	AF155685	EU984276	EU977484
Ophiostoma bicolor	DQ268604	AY497512	DQ268635
Ophiostoma canum	AJ538342	EU984277	EU977485
Ophiostoma floccosum	AJ538343	AF139810	AY789142
Ophiostoma ips	AY172022	AY172021	GU170412
Ophiostoma macrosporum CBS367.53	EU984290	EU984257	EU977465
Ophiostoma montium CBS15178	AY194947	EU984278	AY194963
Ophiostoma montium CBS435.34	EU984289	AY858657	EU977464
Ophiostoma novo-ulmi CMW10573	DQ294375		FJ430508
Ophiostoma piceae	AJ538341	AB007663	AY305698
Ophiostoma pulvinisporum CMW9022	DQ294380		EU977487
Ophiostoma quercus	DQ294376	AF234835	AY789157
Ophiostoma setosum	AF128929		AY305703
Ophiostoma stenoceras CMW3202	DQ294350	FJ176850	DQ296074
Ophiostoma tingens CBS366.53	EU984293	EU984258	EU977468
Ophiostoma ulmi	DQ368627	M83261	EU977489
Ophiostomataceae sp. TR25	EU984281	EU984251	EU977457
Penicillium expansum	U15483	DQ912698	AF003248
Petriella setifera CBS385.87	AF027666	EU984280	EU977491
PL1001[a]	KJ909293[d]	KJ909294[d]	KJ909295[d]
PL1004[b]	KJ909296[d]	KF026302	KJ909297[d]
PL1635	KJ909308[d]	KJ909309[d]	KJ909310[d]
Raffaelea albimanens CBS271.70	EU984296	EU984259	EU977471
Raffaelea amasae CBS116694	EU984295	AY858660	EU977470
Raffaelea ambrosiae CBS185.64	EU984297	AY497518	EU977472
Raffaelea arxii CBS273.70	EU984298	AY497519	
Raffaelea brunnea CBS378.68	EU984284	AY858654	EU977460
Raffaelea canadensis CBS168.66	EU984299	AY858665	EU977473
Raffaelea canadensis CBS805.70	EU984291	AY858658	EU977466
Raffaelea ellipticospora	HQ688664	KJ909299[c,d]	KJ909298[c,d]
Raffaelea fusca C2394[c]	EU177449	KJ909300[d]	KJ909301[d]
Raffaelea gnathotrichi CBS379.68	EU177460	AY858655	
Raffaelea lauricola	EU123077	EU123076	
Raffaelea lauricola PL159[b]	KJ909303[d]	EU257806	KJ909302[d]
Raffaelea montetyi	EU984301	AY497520	EU977475

Table 1 – (*continued*)

Taxon isolate	Accession		
	LSU	SSU	BT
Raffaelea montetyi PC06.001	JF909540	JF909512	
Raffaelea quercivora MAFF410918	AB496454	AB496428	GQ225691
Raffaelea quercus-mongolicae KACC44405		GQ225700	GQ225688
Raffaelea santoroi CBS399.67	EU984302	EU984261	EU977476
Raffaelea scolytodis CCF3572	AM267270	AM267261	
Raffaelea subalba C2401[c]	EU177443	KJ909304[d]	KJ909305[d]
Raffaelea subfusca C2335[c]	EU177450	KJ909306[d]	KJ909307[d]
Raffaelea sulcati CBS806.70	EU177462	AY858666	EU977477
Raffaelea sulphurea CBS380.68	EU984292	EU170272	EU977467
Raffaelea tritirachium CBS726.69	EU984303	EU984262	EU977478
S21		KJ909314[d]	
S22		KJ909311[d]	
S28		KJ909312[d]	
S31		KJ909313[d]	
S32		KJ909315[d]	
Sporothrix humicola CMW7618	EF139114		EF139100
Sporothrix schenckii	DQ294353	M85053	DQ296076
Sporothrix schenckii CMW7614	DQ294352		AY280477
Taphrina populina CBS337.55	AF492050	D14165	AF170968
Xylaria sp.	AY327481	U32417	AY951763

a Isolate UCR 1073 from Eskalen & McDonald (2011).
b From authors collections.
c From Dr. T. C. Harrington Iowa State University.
d Sequenced in this study.

The ML analyses were conducted at the University of Florida High Performance Computing Center (HPC) using RAxML version 7.3.5 using the GTRGAMMAI model, as determined by JModelTest, with 100 distinct starting trees and 1000 bootstrap analyses (BS) (Posada 2008; Stamatakis 2006). Gene sequences (LSU, SSU, BT) missing from isolates were treated as missing data then concatenated to form the combined dataset with 1849 characters total. The combined dataset was analysed using ML, as described above, with each gene in a separate partition.

The MP analysis was conducted using PAUP* 4.0a129 with gaps treated as missing data, a heuristic search with ten random stepwise addition replicates, and TBR (Swofford 2003). Branches with zero branch lengths were collapsed, and support was assessed by BS analysis using 1000 MP heuristic searches using TBR. The Bayesian Inference (BI) analysis was conducted at the HPC using MrBayes 3.2.1 using the GTR+I+G model with all parameters unlinked (adapted from JModelTest), each gene in a separate character set, and 5 million generations that were sampled every 1000 generations (Ronquist *et al.* 2012). The first 5000 trees were discarded as burn-in, as determined using Tracer 1.4, and the remaining 15 002 trees were used to calculate the posterior probabilities (PP) and construct the majority-rule consensus tree using MrBayes (Rambaut & Drummond 2007).

To test for monophyly of *Raffaelea*, Bayes factors (BF) were calculated by first conducting a BI analysis, as described above, with the addition of a constraint that the *Raffaelea* taxa form a single clade. BFs were then calculated using the harmonic mean from MrBayes and the BF from Tracer (Kass & Raftery 1995; Rambaut & Drummond 2007; Ronquist *et al.* 2012). Expected likelihood weight (ELW) and Shimodaira-Hasegawa (SH) tests were conducted in RAxML, as described above, with the addition of a monophyletic *Raffaelea* constraint tree (Stamatakis 2006).

An additional ML analysis was performed to determine the placement of undescribed isolates. To do this, sequences from seven isolates were included in the concatenated dataset: five (S21, S22, S28, S31, S32) from nutmeg, *Myristica fragrans*, with wilt symptoms in Grenada, one (PL1001, strain UCR 1073 GenBank Accession JF327799 from Eskalen & McDonald (2011) from avocado with wilt symptoms in California), and one (PL1635) associated with a pine-specific ambrosia beetle in Thailand. Only SSU sequences were available for the five isolates from Grenada, whereas SSU, LSU, and BT sequences were available for the remaining undescribed isolates. DNA sequence alignments and phylogenetic trees were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S15908).

Results

After running for 2 h, the first PHT, with Maxtree set to auto increase, was still on replicate 1a and had 500 532, and increasing, trees remaining to swap and was aborted. The inability of the PHT to reach completion was not surprising because the MP analysis of the LSU dataset resulted in 20 700 equally parsimonious trees (Fig S5). The next PHT analyses using Maxtree = 500, yielded *P* values of 0.01 and 0.073 (TBR with 100 replicates, and NNI with 1000 replicates, respectively). Results from the PHT indicate the genes might be incongruent

but are questionable because of the limited search strategies that were employed so the analysis could be completed effectively. For these reasons and other shortcomings of the PHT, as noted by Hipp et al. (2004) and references therein, we believe the PHT results do not provide sufficient evidence not to combine the datasets. The ML analyses of the individual genes showed weak support for both deeper nodes and terminal branches but the general topologies were similar (Fig S2–S4). The most notable differences were the placements of *Ceratocystiopsis* and *Fragosphaeria*, which probably contributed to the incongruent PHT. However, following similar conclusions by Massoumi Alamouti et al. (2009), we accepted that the gene histories were sufficiently similar to combine the data and we present results from both the combined and individual datasets (Figs 1 and 2, Fig S2–S4).

Taxa in the Ophiostomatales formed a highly supported clade with 100, 1, and 99 ML BS, BI PP, and MP BS values, respectively. All three analyses strongly supported placement of *Ceratocystiopsis* and *Fragosphaeria* in the Ophiostomatales; however, they could not be placed relative to the other genera because the individual gene phylogenies had different topologies (Fig 1, Fig S2–S4). The *Ophiostoma sensu lato* clade was well supported with 88, 1, and 77 ML BS, BI PP, and MP BS values, respectively. *Raffaelea* fell into two clades, one of which included *Raffaelea amasae*, *Raffaelea sulphurea*, *Raffaelea quercus-mongolicae*, *Raffaelea quercivora*, *Raffaelea montetyi*, and *Esteya vermicola* (97, 1, and 89 ML BS, BI PP, and MP BS values, respectively) within the *Leptographium sensu lato* clade (87, 1, 67, ML BS, BI PP, and MP BS values, respectively). The second *Raffaelea* clade contained *Raffaelea brunnea*, *Raffaelea lauricola*, *Raffaelea scolytodis*, *Raffaelea arxii*, *Raffaelea gnathotrichi*, *Raffaelea fusca*, *Raffaelea subfusca*, *Raffaelea ellipticospora*, *R. ambrosiae* (type species for the genus), *Raffaelea canadensis*, *Raffaelea albimanens*, *Raffaelea subalba*, *Raffaelea tritirachium*, *Raffaelea santoroi*, and *Raffaelea sulcati* (93, 1, and 87 ML BS, BI PP, and MP BS values, respectively) and was sister to *Leptographium sensu lato*. The placement of *Fragosphaeria* was disregarded due to the incongruence of the different loci and the consequent uncertainty in its placement.

The log likelihood values from the ML unconstrained and the monophyletic *Raffaelea* constraint analyses were (−15 790.81 and −15 822.69) and for the BI analyses were (−15 943.84 and −15 973.97 from Tracer) and (−15 960.43 and −15 997.19 from MrBayes), respectively. Although the ELW test indicated that the monophyletic constrained hypothesis was significantly worse than the unconstrained hypothesis (polyphyletic *Raffaelea*) (0.954 PP), the SH test did not find a significant difference between the hypotheses at alpha < 0.05. The BFs were greater than 30 for both methods used, indicating very strong support for the polyphyletic *Raffaelea* hypothesis (Kass & Raftery 1995).

The ML analysis of the unidentified isolates provided evidence for six new taxa, and supported previous indications that isolate TR25 represented a distinct taxon (Massoumi Alamouti et al. 2009) (Fig 2). In the *Leptographium sensu lato* clade, isolate S28 was close to *R. sulphurea*, and isolates S31 and S32 were close to *R. amasae*. In the *Raffaelea sensu stricto* clade, S21 and S22 were close to PL1004 (see Dreaden et al. 2014 for more information on this isolate) and *R. brunnea*, PL1001 was near *R. canadensis*, and PL1635 was near *R. scolytodis*.

Discussion

The ML analyses of the individual gene datasets along with the ML, BI, and MP analyses of the combined dataset all indicated that *Raffaelea*, as it is currently defined, is polyphyletic. *Esteya vermicola* together with *Raffaelea amasae*, *Raffaelea sulphurea*, *Raffaelea quercus-mongolicae*, *Raffaelea quercivora*, and *Raffaelea montetyi* formed a strongly supported clade in *Leptographium sensu lato* (Fig 1, Fig S2–S4). The remaining *Raffaelea* spp. resided in a second clade sister to *Leptographium sensu lato*, also with strong statistical support. Of the three tests used to consider monophyly in *Raffaelea*, only the SH test indicated that the constrained tree did not differ from the unconstrained tree. This is not surprising as the SH test has been shown to be conservative (Czarna et al. 2006; Shimodaira & Hasegawa 1999; Strimmer & Rambaut 2002). Taken as a whole, the evidence suggests that *Raffaelea* needs to be reevaluated and that *Leptographium sensu lato* should be included in this reevaluation.

This study recognizes *Raffaelea brunnea*, *Raffaelea lauricola*, *Raffaelea scolytodis*, *Raffaelea arxii*, *Raffaelea gnathotrichi*, *Raffaelea fusca*, *Raffaelea subfusca*, *Raffaelea ellipticospora*, *Raffaelea ambrosiae*, *Raffaelea canadensis*, *Raffaelea albimanens*, *Raffaelea subalba*, *Raffaelea tritirachium*, *Raffaelea santoroi*, and *Raffaelea sulcati* as *Raffaelea sensu stricto*. *Raffaelea amasae*, *R. sulphurea*, *R. quercus-mongolicae*, *R. quercivora*, and *R. montetyi* should be removed from *Raffaelea*, but their correct placement remains unclear at this time. Whether they should be placed in *Leptographium sensu lato* or accommodated in a reinstated *Dryadomyces* with *Dryadomyces amasae* as the type species will require additional research. In particular, a phylogenetic study that includes all, or most, *Leptographium sensu lato* and *Raffaelea* taxa is recommended.

Massoumi Alamouti et al. (2009) noted, referencing work by Cassar & Blackwell (1996) and Farrell et al. (2001), that SSU-based phylogenies indicated that both *Ambrosiella* and *Raffaelea* are polyphyletic. This led these authors to suggest that the similar morphologies of the two genera and their intimate associations with ambrosia beetles arose more than once in each genus. The ambrosial habit in beetles is also polyphyletic and has arisen at least seven times (Farrell et al. 2001). The multiple origins of both ambrosial fungi, including *Raffaelea*, and the beetles with which they are associated suggests that these relationships should not be used to define *Raffaelea*.

The ML phylogenies of individual gene datasets and ML, BI, and MP phylogenies of the combined dataset in the present study show that *Raffaelea* is polyphyletic. This contradicts the MP results of Harrington et al. (2010) based on LSU data but is consistent with those based on the SSU data. These discrepancies could be due to differences in taxon sampling, the loci that were used, and the methodologies used to define these relationships (MP *vs.* ML). Although the effect of taxon sampling was not studied, the latter factors were shown to be significant, as a MP analysis of LSU data in the present study also placed *Raffaelea* spp. in a single clade (Fig S5). Thus, it appears that the previous conclusion (Harrington et al. 2010) that *Raffaelea* is monophyletic was an artifact of the MP analysis and LSU dataset that they used.

Fig 1 – *Raffaelea* ML phylogeny from the combined, LSU, SSU, and BT dataset. Clade support values are ML bootstrap percentages with BI posterior probabilities >0.9 and MP bootstrap percentages >70 % for selected clades shown as bars above and below the branches, respectively. Type species for select genera are indicated in blue and isolates missing gene sequences have the genes that were used listed in red. *Raffaelea* isolates are highlighted with red bars and *Leptographium sensu lato* with a blue bar (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The ML analyses suggest that the nine unidentified isolates included in this study contain seven undescribed taxa (Fig 2). These will be described elsewhere, as additional isolates become available. The results also provide a strong indication that there are many more new species of *Raffaelea* that remain to be identified. Clearly, care should be taken when new isolates of *Raffaelea* are identified and diagnostic and detection methods are designed. For example, isolate PL1004 had been identified as *Raffaelea lauricola*, based on SSU data, but was shown later to be non-pathogenic and is now considered to

Fig 2 – *Raffaelea* ML phylogeny with unidentified isolates, bold, from the combined, LSU, SSU, and BT dataset. Clade support values are ML bootstrap percentages. Notice there is support for seven new taxa 1. S28, 2. S31 and S32, 3. S21 and S22, 4. PL1004, 5. *Ophiostomataceae* sp. TR25, 6. PL1001, and 7. PL1635. Type species for select genera are labelled in blue and isolates missing gene sequences have the genes that are available listed in red. *Raffaelea* isolates are highlighted with red bars and *Leptographium sensu lato* with a blue bar (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

be a new species (Dreaden *et al.* 2014). Additionally, the *R. lauricola* detection method developed by Jeyaprakash *et al.* (2014) utilizes a portion of the LSU where PL1004 and *R. lauricola* have 100 % sequence homology, implying that the method will likely detect PL1004 and *R. lauricola* equally well and thus resulting in false positives. Likewise, SSU data were used to identify isolate PL1001 as *Raffaelea canadensis* (Eskalen & McDonald 2011), which was shown in the present study to differ from that species. A more detailed study that includes additional isolates of the putative new taxa is needed

to formally describe them as new species. The BS support for this analysis was lower for many clades when compared to the analysis not including the unknown isolates. This was probably due to the uncertain placement of the isolates from Grenada for which only SSU sequences were available (Figs 1 and 2).

The ITS region has been widely used for fungal diagnostics, phylogenetics and has been proposed as the universal DNA barcode marker for Fungi (Schoch *et al.* 2012). Unfortunately, the locus is notoriously difficult to utilize in *Raffaelea* (Harrington *et al.* 2011; Jeyaprakash *et al.* 2014). We were able to produce PCR amplicons, after much trial and error, for many *Raffaelea* spp. but only one high quality ITS sequence could be generated and this sequence along with those from GenBank could not be unambiguously aligned (Fig S1). Due to these difficulties, the ITS locus was not used to discern the phylogeny of *Raffaelea* spp. in this study. Jeyaprakash *et al.* (2014) were able to partially characterize the ITS for a *R. lauricola* isolate, after considerable modification to their sequencing methodology, and when aligned with the *R. lauricola* ITS2 sequence generated here has 15 bp differences, GenBank Accessions KJ909303 and KF515711 respectively. It would be interesting to sequence multiple cloned ITS PCR amplicons from multiple *R. lauricola* isolates to determine the prevalence of intraspecific and intragenomic ITS variants.

Acknowledgements

Funding for this project was provided by the U. S. Department of Agriculture AFRI Biosecurity Program Project Number 2010-85605-20537 and National Institute of Food and Agriculture grant 2009-51181-05915. We thank Dr. Tom Harrington, Dr. Akif Eskalen, and Craig Bateman for providing isolates and Adam Black, Dr. Robert Blanchette, Dr. Tuan Duong, members of the University of Florida Forest Pathology Laboratory and the University of Pretoria Forestry and Agricultural Biotechnology Institute for their assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2014.09.001.

REFERENCES

Arx JA von, Hennebert GL, 1965. Deux champignons ambrosia. *Mycopathologia et Mycologia Applicata* **25**: 309–315.

Batra LR, 1967. Ambrosia fungi: a taxonomic revision and nutritional status of some species. *Mycologia* **59**: 979–1017.

Cassar S, Blackwell M, 1996. Convergent origins of ambrosia fungi. *Mycologia* **88**: 596–601.

Czarna A, Sanjuán R, González-Candelas F, Wróbel B, 2006. Topology testing of phylogenies using least squares methods. *BMC Evolutionary Biology* **6**: 105.

De Beer ZW, Seifert KA, Wingfield MJ, 2013a. The ophiostomatoid fungi: their dual position in the Sordariomycetes. In: Seifert KA, De Beer ZW, Wingfield MJ (eds), *The Ophiostomatoid Fungi: expanding frontiers CBS Biodiversity Series*, vol. 12. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, pp. 1–19.

De Beer ZW, Seifert KA, Wingfield MJ, 2013b. A nomenclator for ophiostomatoid genera and species in the *Ophiostomatales* and *Microascales*. In: Seifert KA, De Beer ZW, Wingfield MJ (eds), *The Ophiostomatoid Fungi: expanding frontiers CBS Biodiversity Series*, vol. 12. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, pp. 245–322.

De Beer ZW, Wingfield MJ, 2013. Emerging lineages in the *Ophiostomatales*. In: Seifert KA, De Beer ZW, Wingfield MJ (eds), *The Ophiostomatoid Fungi: expanding frontiers CBS Biodiversity Series*, vol. 12. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, pp. 21–46.

Dreaden TJ, Davis JM, Harmon CL, Ploetz RC, Palmateer AJ, Soltis PS, Smith JA, 2014. Development of multilocus PCR assays for *Raffaelea lauricola*, causal agent of laurel wilt disease. *Plant Disease* **98**: 379–383.

Duong TA, de Beer ZW, Wingfield BD, Wingfield MJ, 2012. Phylogeny and taxonomy of species in the *Grosmannia serpens* complex. *Mycologia* **104**: 715–732. http://dx.doi.org/10.3852/11-109.

Eskalen A, McDonald V, 2011. First report of *Raffaelea canadensis* causing laurel wilt disease symptoms on avocado in California. *Plant Disease* **95**: 9–1189.

Farrell BD, Sequeira ASO, Meara BC, Normark BB, Chung JH, Jordal BH, 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution* **55**: 2011–2027.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Gebhardt H, Oberwinkler F, 2005. Conidial development in selected ambrosial species of the genus *Raffaelea*. *Antonie van Leeuwenhoek* **88**: 61–66.

Gebhardt H, Weiss M, Oberwinkler F, 2005. *Dryadomyces amasae*: a nutritional fungus associated with ambrosia beetles of the genus Amasa (Coleoptera: Curculionidae, Scolytinae). *Mycological Research* **109**: 687–696.

Glass NL, Donaldson GC, 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323–1330.

Harrington TC, Yun HY, Lu SS, Goto H, Aghayeva DN, Fraedrich SW, 2011. Isolations from the redbay ambrosia beetle, *Xyleborus glabratus*, confirm that the laurel wilt pathogen, *Raffaelea lauricola*, originated in Asia. *Mycologia* **103**: 1028–1036.

Harrington TC, Aghayeva DN, Fraedrich SW, 2010. New combinations in *Raffaelea*, *Ambrosiella*, and *Hyalorhinocladiella*, and four new species from the redbay ambrosia beetle, *Xyleborus glabratus*. *Mycotaxon* **111**: 337–361.

Harrington TC, Fraedrich SW, Aghayeva DN, 2008. *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon* **104**: 399–404.

Hipp AL, Hall JC, Sytsma KJ, 2004. Congruence versus phylogenetic accuracy: revisiting the incongruence length difference test. *Systematic Biology* **53**: 81–89.

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, 70 co-authors, 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* **443**: 818–822.

Jeyaprakash A, Davison DA, Schubert TS, 2014. Molecular detection of the laurel wilt fungus, *Raffaelea lauricola*. *Plant Disease* **98**: 559–654.

Jones KG, Blackwell M, 1998. Phylogenetic analysis of ambrosial species in the genus *Raffaelea* based on 18S rDNA sequences. *Mycological Research* **102**: 661–665.

Justesen AF, Ridout CJ, Hovmoller MS, 2002. The recent history of *Puccinia striiformis* f.sp. *tritici* in Denmark as revealed by disease incidence and AFLP markers. *Plant Pathology* **51**: 13–23.

Kass RE, Raftery AE, 1995. Bayes factors. *Journal of the American Statistical Association* **90**: 773–795.
Kim KH, Choi YJ, Seo ST, Shin HD, 2009. *Raffaelea quercus-mongolicae* sp. nov. associated with *Platypus koryoensis* on oak in Korea. *Mycotaxon* **110**: 189–197.
Kubono T, Ito S, 2002. *Raffaelea quercivora* sp. Novo associated with mass mortality of Japanese oak, and the ambrosia beetle (*Platypus quercivorus*). *Mycoscience* **43**: 255–260.
Massoumi Alamouti S, Tsui CKM, Breuil C, 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. *Mycological Research* **113**: 822–835.
O'Donnell K, 1993. *Fusarium* and its near relatives. In: Reynolds R, Taylor JW (eds), *The Fungal Holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*. CBA International, Wallingford, United Kingdom, pp. 225–233.
Ploetz RC, Hulcr J, Wingfield MJ, de Beer ZW, 2013. Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Disease* **97**: 856–872.
Ploetz RC, Pena JE, Smith JA, Dreaden TJ, Crane JH, Schubert T, Dixon W, 2011. Laurel wilt, caused by *Raffaelea lauricola*, is confirmed in Miami-Dade County, Center of Florida's Commercial Avocado Production. *Plant Disease* **95**: 1589.
Posada D, 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.
Rambaut A, Drummond AJ, 2007. *Tracer* 1.4 Available at: http://beast.bio.ed.ac.uk/Tracer
Ronquist F, Teslenko M, van der Mark P, Ayres D, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP, 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**: 539–542.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque A, Chen WFungal Barcode Consortium, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 6241–6246. http://dx.doi.org/10.1073/pnas.1117018109.
Shimodaira H, Hasegawa M, 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology Evolution* **16**: 1114–1116.
Slippers B, Boissin E, Phillips AJ, Groenewald JZ, Lombard L, Wingfield MJ, Postma A, Burgess T, Crous PW, 2013. Phylogenetic lineages in the Botryosphaeriales: a systematic and evolutionary framework. *Studies in Mycology* **76**: 31–49. http://dx.doi.org/10.3114/sim0020.
Stamatakis A, 2006. RAxML-VI-HPC: Maximum Likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.
Strimmer K, Rambaut A, 2002. Inferring confidence sets of possibly misspecified gene trees. *Proceedings of the Royal Society London Biological Sciences* **269**: 137–142.
Swofford DL, 2003. *PAUP* 4.0: phylogenetic analysis using parsimony (*and other methods)*. Sinauer Associates, Sunderland, Massachusetts.
Vilgalys R, Hester M, 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.
Wingfield BD, Van Wyk M, Roos H, Wingfield MJ, 2013. Ceratocystis: emerging evidence for discrete generic boundaries. In: Seifert KA, De Beer ZW, Wingfield MJ (eds), *The Ophiostomatoid Fungi: expanding frontiers CBS Biodiversity Series*, vol. 12. CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands, pp. 57–64.
White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Inis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego, pp. 315–322.