

Phylogeny of ambrosia beetle symbionts in the genus *Raffaelea*

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Abstract

The genus *Raffaelea* was established in 1965 when the type species, *R. 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 *R. quercivora*, *R. quercus-mongolicae*, and *R. 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.

Keywords

Raffaelea sensu stricto; Ophiostomatales; internal transcribed spacer region; ITS; ambrosia fungi; wilt pathogens

Research highlights

- *Raffaelea* as it is currently defined is not monophyletic.
- *Raffaelea sensu stricto* is defined.
- *Raffaelea* spp. isolates with unclear affinities are identified.

1. 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 *R. lauricola*, *R. quercivora*, and *R. quercus-mongolicae* are serious pathogens that can cause significant damage to forests and fruit crops (Kim et al. 2009, Kubono and 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 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 and Hennebert (1965) to accommodate *R. 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 and 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 *D. amasae* (= *R. 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 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*, *R. 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*, *R. montetyi*, *R. 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.

2. Materials and Methods

2.1. 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 analyzed, including 9 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).

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

Taxon Isolate	Accession		
	LSU	SSU	BT
<i>Ambrosiella ferruginea</i> CBS408.68	EU984285	EU984254	EU977461
<i>Ambrosiella ferruginea</i> JB13	EU984286	EU984255	EU977462
<i>Ambrosiella hartigii</i> CBS404.82	EU984288	EU984256	EU977463
<i>Ambrosiella xylebori</i> CBS110.61	EU984294	AY858659	EU977469
<i>Ceratocystiopsis manitobensis</i> UM237	DQ268607	EU984266	DQ268638
<i>Ceratocystiopsis minuta</i> CBS463.77	DQ268615	EU984267	EU977481
<i>Ceratocystiopsis minuta-bicolor</i> CBS635.66	DQ268616	EU984268	EU977482
<i>Ceratocystis adiposa</i> CBS600.74	EU984304	EU984263	EU977479
<i>Ceratocystis coerulescens</i> CL13-12	AY214000	EU984264	AY140945
<i>Ceratocystis moniliformis</i> CBS155.62	EU984305	EU984265	EU977480
<i>Claviceps fusiformis</i> ATCC26019	U17402	DQ522539	AF263569
<i>Daldinia concentrica</i>	U47828	U32402	FJ185285
<i>Epichloe typhina</i>	U17396	AB105953	X52616
<i>Esteya vermicola</i> CBS115803	EU668903		FJ490552
<i>Fragosphaeria purpurea</i> CBS133.34	AF096191	AF096176	
<i>Fragosphaeria reniformis</i> CBS134.34	AB189155	AB278193	
<i>Grosmannia abiocarpa</i> MUCL18351	AJ538339	EU984269	DQ097857
<i>Grosmannia clavigera</i> ATCC18086	AY544613	EU984270	AY263194
<i>Grosmannia cucullata</i>	AJ538335	AY497513	EU977483
<i>Grosmannia penicillata</i>	DQ097851	AY858662	DQ097861
<i>Grosmannia piceiperda</i>	AY707209	AY497514	AY707195
<i>Grosmannia serpens</i>	DQ294394	AY497516	AY707188
<i>Leptographium abietinum</i> DAOM60343	DQ097852	EU984271	AY263182
<i>Leptographium fruticetum</i> DAOM234390	DQ097848	EU984272	DQ097855
<i>Leptographium longiclavatum</i>	AY816686	EU984273	AY288934
<i>Leptographium lundbergii</i> UAMH9584	AY544603	EU984274	AY263184
<i>Leptographium terebrantis</i> UAMH9722	AY544606	EU984275	AY263192
<i>Microascus cirrosus</i> CBS217.31	AF275539	EU984279	EU977490
<i>Ophiostoma abietinum</i>	AF155685	EU984276	EU977484
<i>Ophiostoma bicolor</i>	DQ268604	AY497512	DQ268635
<i>Ophiostoma canum</i>	AJ538342	EU984277	EU977485
<i>Ophiostoma floccosum</i>	AJ538343	AF139810	AY789142
<i>Ophiostoma ips</i>	AY172022	AY172021	GU170412
<i>Ophiostoma macrosporum</i> CBS367.53	EU984290	EU984257	EU977465
<i>Ophiostoma montium</i> CBS15178	AY194947	EU984278	AY194963

<i>Ophiostoma montium</i> CBS435.34	EU984289	AY858657	EU977464
<i>Ophiostoma novo-ulmi</i> CMW10573	DQ294375		FJ430508
<i>Ophiostoma piceae</i>	AJ538341	AB007663	AY305698
<i>Ophiostoma pulvinisporum</i> CMW9022	DQ294380		EU977487
<i>Ophiostoma quercus</i>	DQ294376	AF234835	AY789157
<i>Ophiostoma setosum</i>	AF128929		AY305703
<i>Ophiostoma stenoceras</i> CMW3202	DQ294350	FJ176850	DQ296074
<i>Ophiostoma tingens</i> CBS366.53	EU984293	EU984258	EU977468
<i>Ophiostoma ulmi</i>	DQ368627	M83261	EU977489
<i>Ophiostomataceae</i> sp. TR25	EU984281	EU984251	EU977457
<i>Penicillium expansum</i>	U15483	DQ912698	AF003248
<i>Petriella setifera</i> 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
<i>Raffaelea albimanens</i> CBS271.70	EU984296	EU984259	EU977471
<i>Raffaelea amasae</i> CBS116694	EU984295	AY858660	EU977470
<i>Raffaelea ambrosiae</i> CBS185.64	EU984297	AY497518	EU977472
<i>Raffaelea arxii</i> CBS273.70	EU984298	AY497519	
<i>Raffaelea brunnea</i> CBS378.68	EU984284	AY858654	EU977460
<i>Raffaelea canadensis</i> CBS168.66	EU984299	AY858665	EU977473
<i>Raffaelea canadensis</i> CBS805.70	EU984291	AY858658	EU977466
<i>Raffaelea ellipticospora</i>	HQ688664	KJ909299 ^{cd}	KJ909298 ^{cd}
<i>Raffaelea fusca</i> C2394 ^c	EU177449	KJ909300 ^d	KJ909301 ^d
<i>Raffaelea gnathotrichi</i> CBS379.68	EU177460	AY858655	
<i>Raffaelea lauricola</i>	EU123077	EU123076	
<i>Raffaelea lauricola</i> PL159 ^b	KJ909303 ^d	EU257806	KJ909302 ^d
<i>Raffaelea montetyi</i>	EU984301	AY497520	EU977475
<i>Raffaelea montetyi</i> PC06.001	JF909540	JF909512	
<i>Raffaelea quercivora</i> MAFF410918	AB496454	AB496428	GQ225691
<i>Raffaelea quercus-mongolicae</i> KACC44405		GQ225700	GQ225688
<i>Raffaelea santoroi</i> CBS399.67	EU984302	EU984261	EU977476
<i>Raffaelea scolytodis</i> CCF3572	AM267270	AM267261	
<i>Raffaelea subalba</i> C2401 ^c	EU177443	KJ909304 ^d	KJ909305 ^d
<i>Raffaelea subfusca</i> C2335 ^c	EU177450	KJ909306 ^d	KJ909307 ^d
<i>Raffaelea sulcati</i> CBS806.70	EU177462	AY858666	EU977477
<i>Raffaelea sulphurea</i> CBS380.68	EU984292	EU170272	EU977467
<i>Raffaelea tritirachium</i> CBS726.69	EU984303	EU984262	EU977478
S21		KJ909314 ^d	
S22		KJ909311 ^d	
S28		KJ909312 ^d	
S31		KJ909313 ^d	
S32		KJ909315 ^d	

<i>Sporothrix humicola</i> CMW7618	EF139114		EF139100
<i>Sporothrix schenckii</i>	DQ294353	M85053	DQ296076
<i>Sporothrix schenckii</i> CMW7614	DQ294352		AY280477
<i>Taphrina populina</i> CBS337.55	AF492050	D14165	AF170968
<i>Xylaria</i> sp.	AY327481	U32417	AY951763

^a isolate UCR1073 from Eskalen and McDonald (2011), ^bfrom authors collections,

^c from Dr. T. C. Harrington Iowa State University, ^d Sequenced in this study,

2.2. 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 and Donaldson 1995, O'Donnell 1993, Vilgalys and 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 Ltd., 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 and 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.

2.3. 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* 4.0a1, 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. 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 data set with 1849 characters total. The combined data set was analyzed 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 10 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 5,000 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 and 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 and Raftery 1995, Rambaut and 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 and 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>).

3. Results

After running for two hours, 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 (Fig 1, 2, 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, 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 *R. amasae*, *R. sulphurea*, *R. quercus-mongolicae*, *R. quercivora*, *R. montetyi*, and *E. vermicola* (97, 1, and 89 ML



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 a red bars and *Leptographium sensu lato* with a blue bar.

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 *R. brunnea*, *R. lauricola*, *R. scolytodis*, *R. arxii*, *R. gnathotrichi*, *R. fusca*, *R. subfusca*, *R. ellipticospora*, *R. ambrosiae* (type species for the genus), *R. canadensis*, *R. albimanens*, *R. subalba*, *R. tritirachium*, *R. santoroi*, and *R. 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 (-15790.81 and -15822.69) and for the BI analyses were (-15943.84 and -15973.97 from Tracer) and (-15960.43 and -15997.19 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 and 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. scolytoidis*.

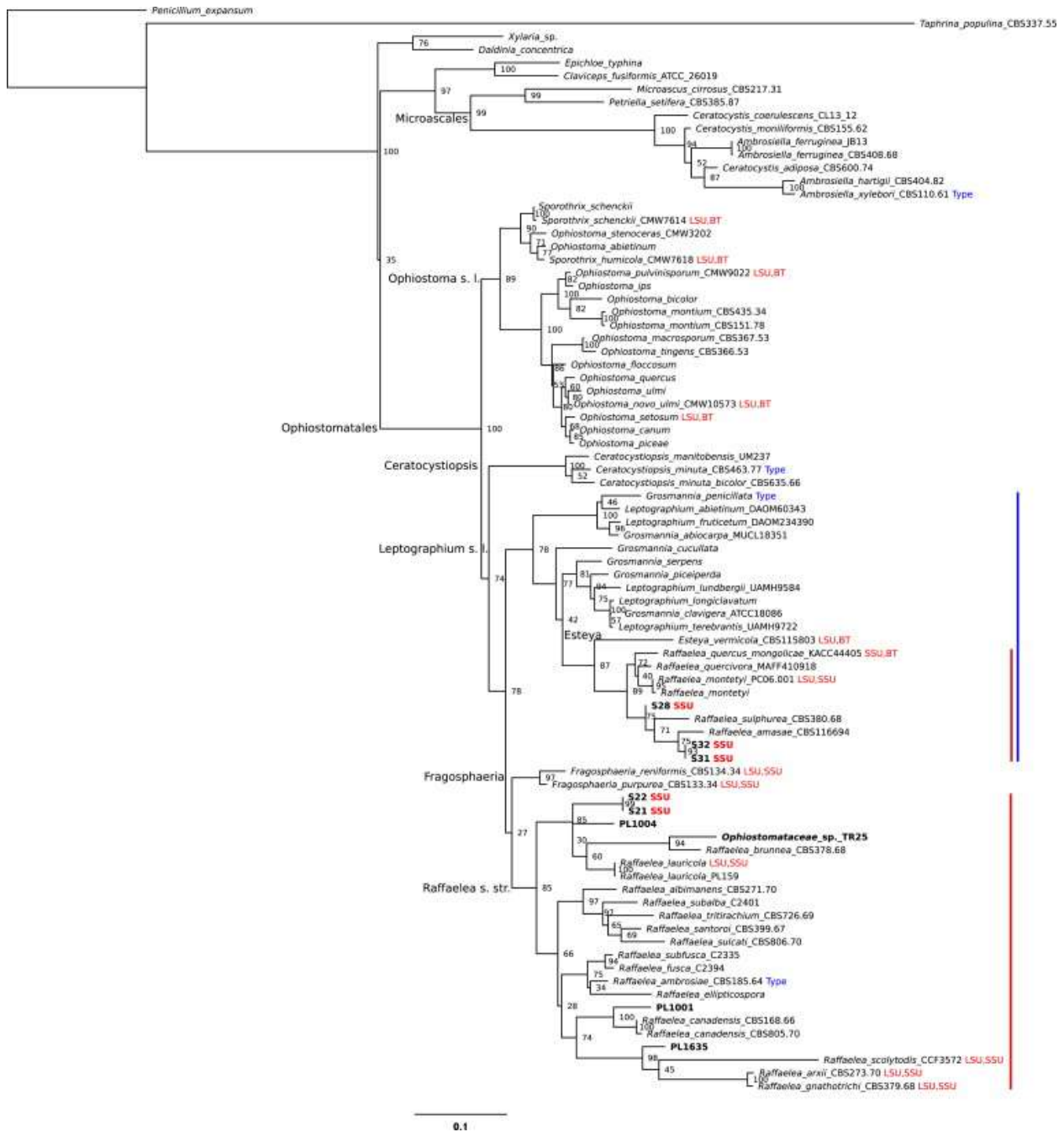


Fig 2. *Raffaelea* ML phylogeny with unidentified isolates, **bold**, from the combined, LSU, SSU, and BT dataset. Clade support values are ML bootstrap percentages. Notice there is support for 7 new taxa 1. S28, 2. S31 and S32, 3. S21 and S22, 4. PL1004, 5. *Ophiostomataceae* sp. TR25, 6. PL1001, and 7. PL1635. Type species for select genera are labeled 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.

4. 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 *R. amasae*, *R. sulphurea*, *R. quercus-mongolicae*, *R. quercivora*, and *R. montetyi* formed a strongly supported clade in *Leptographium sensu lato* (Fig 1, 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 and Hasegawa 1999, Strimmer and 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 *R. brunnea*, *R. lauricola*, *R. scolytodis*, *R. arxii*, *R. gnathotrichi*, *R. fusca*, *R. subfusca*, *R. ellipticospora*, *R. ambrosiae*, *R. canadensis*, *R. albimanens*, *R. subalba*, *R. tritirachium*, *R. santoroi*, and *R. 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 *D. 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 and 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.

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 *R. lauricola*, based on SSU data, but was shown later to be non-

pathogenic and is now considered to 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 *R. canadensis* (Eskalen and 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 (Fig 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.

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Supplementary data

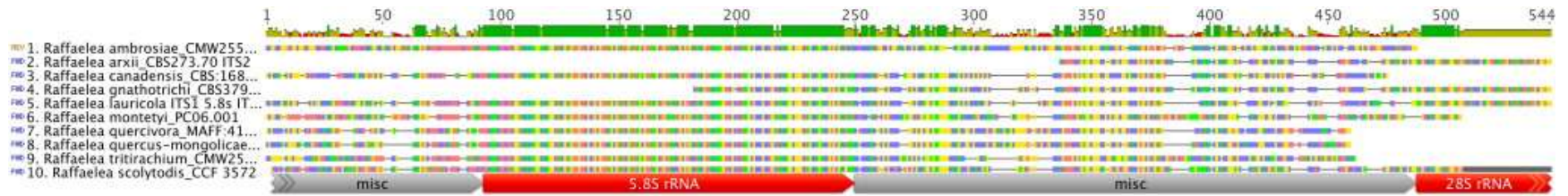


Fig S1. Alignment of the rDNA internal transcribed spacer region, ITS, from 10 *Raffaelea* species. Note that only the 5.8s and portions of the 28s LSU could be aligned unambiguously. The bar graph at the top of the figure indicates the degree of similarity among sequences.

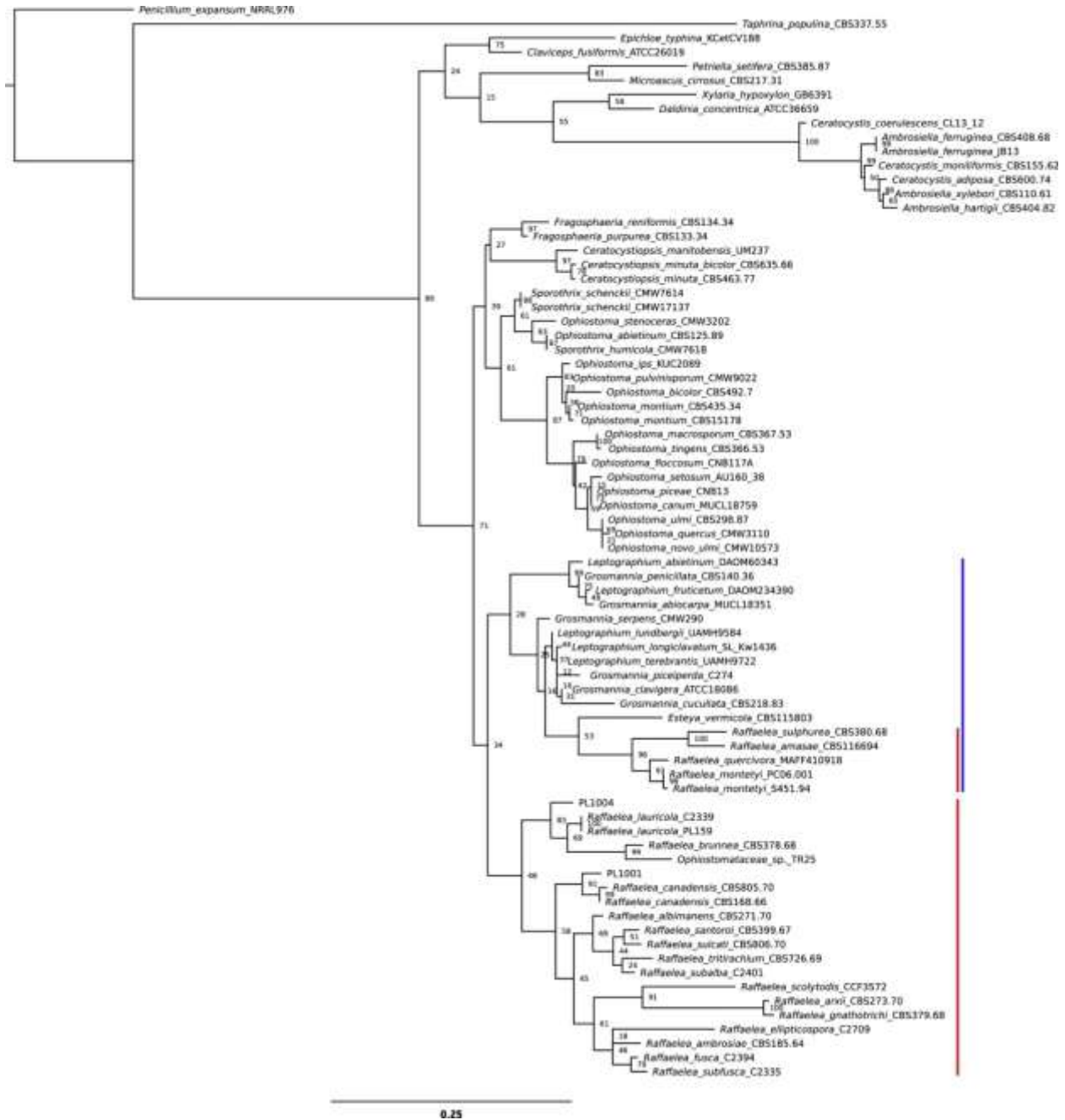


Fig S2. LSU-based phylogeny of *Raffaelea* and related taxa. The bipartition tree from the RAxML maximum likelihood analysis with 1000 bootstrap replicates is shown. *Raffaelea* isolates are highlighted with red bars and *Leptographium sensu lato* with a blue bar to aid comparisons of the individual gene phylogenies (Fig S2–S4).

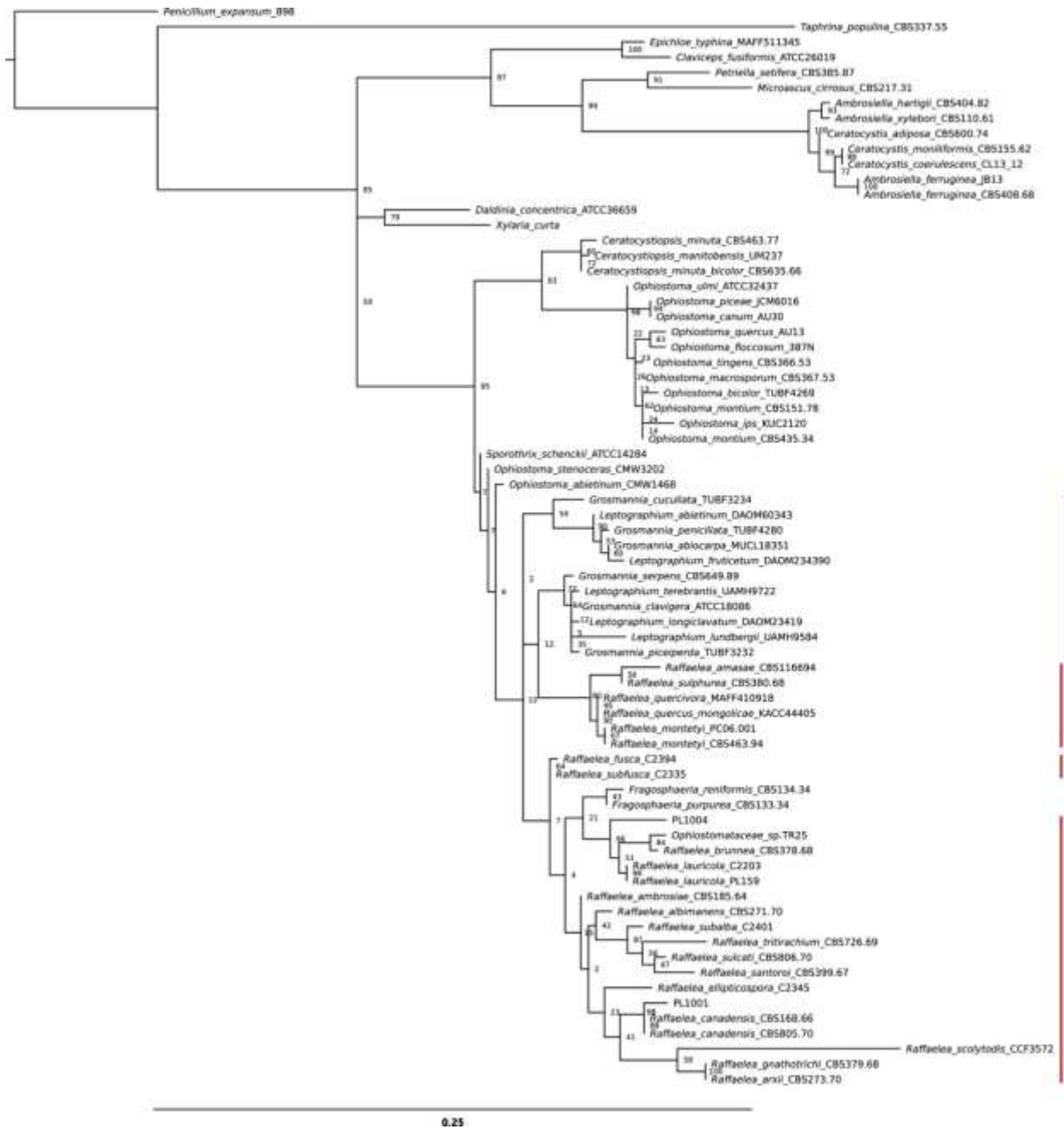


Fig S3. SSU-based phylogeny of *Raffaelea* and related taxa. The bipartition tree from the RAxML maximum likelihood analysis with 1000 bootstrap replicates is shown. *Raffaelea* isolates are highlighted with red bars and *Leptographium sensu lato* with a blue bar to aid comparisons of the individual gene phylogenies (Fig S2–S4).



Fig S4. BT-based phylogeny of *Raffaelea* and related taxa. The bipartition tree from the RAxML maximum likelihood analysis with 1000 bootstrap replicates is shown. *Raffaelea* isolates are highlighted with red bars and *Leptographium sensu lato* with a blue bar to aid comparisons of the individual gene phylogenies (Fig S2–S4).

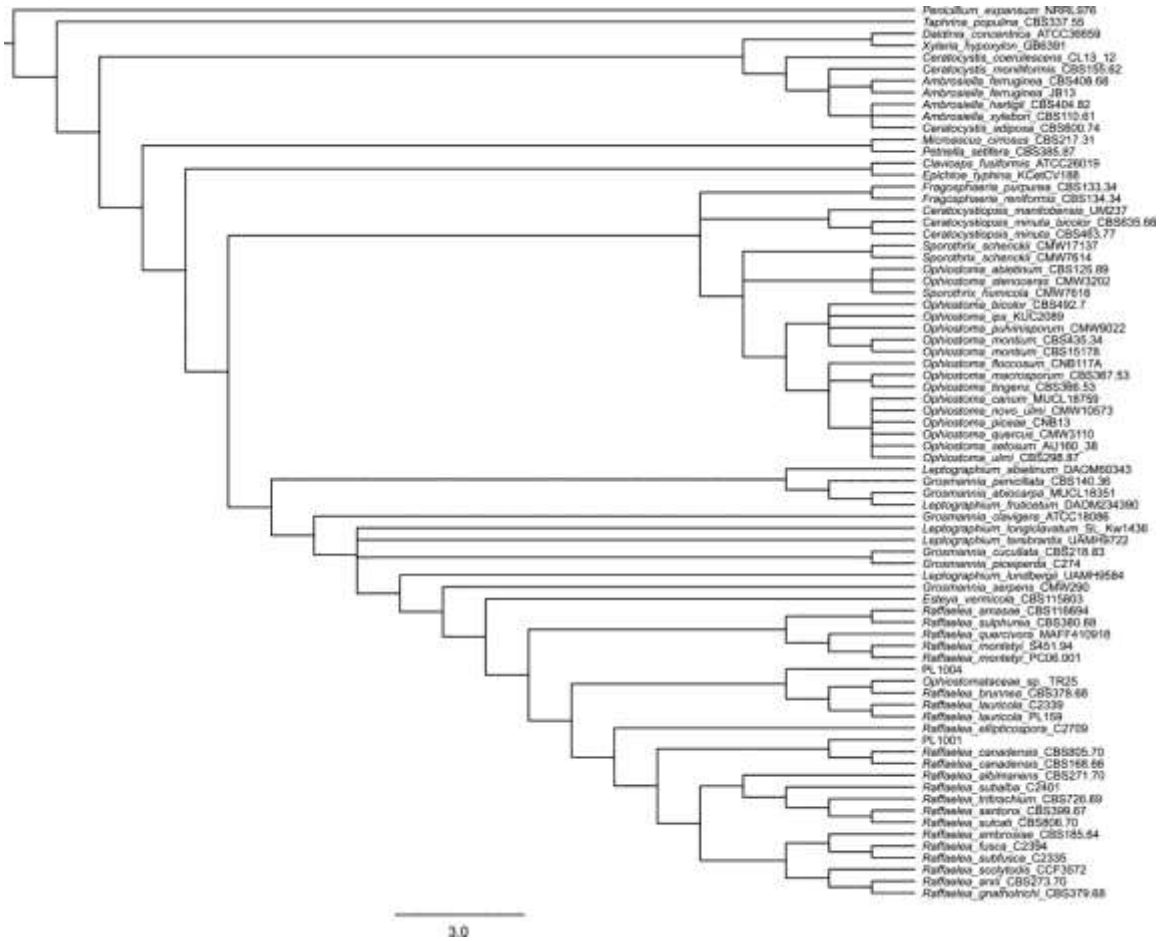


Fig S5. Strict consensus of 20,700 most parsimonious trees using the LSU dataset and PAUP* (465 total characters, 230 were constant, and 195 were parsimony-informative). Gaps were treated as missing data, and the TBR branch swapping algorithm was used (Swofford 2003). This LSU MP analysis found *Raffaelea* as defined by Harrington et al. (2010) to be monophyletic, as did their LSU MP analysis. Where as, the ML (LSU, SSU and BT) analyses (Fig S2-S4) as well as the ML, BI and MP analyses using the combined data sets, in this study, found *Raffaelea* spp. in both the *Raffaelea* and the *Leptographium sensu lato* clades (Fig 1). These findings provide evidence the cause of the incongruence between our findings that *Raffaelea* is not monophyletic, and the Harrington et al. (2010) LSU MP study is because of an interaction between the LSU dataset and MP.