

Know your farmer: Ancient origins and multiple independent domestications of ambrosia beetle fungal cultivars

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Abstract

Bark and ambrosia beetles are highly specialized weevils (Curculionidae) that have established diverse symbioses with fungi, most often from the order Ophiostomatales (Ascomycota, Sordariomycetes). The two types of beetles are distinguished by their feeding habits and intimacy of interactions with their symbiotic fungi. The tree tissue diet of bark beetles is facilitated by fungi, while ambrosia beetles feed solely on fungi that they farm. The farming life history strategy requires domestication of a fungus, which the beetles consume as their sole food source. Ambrosia beetles in the subfamily Platypodinae originated in the mid-Cretaceous (119–88 Ma) and are the oldest known group of farming insects. However, attempts to resolve phylogenetic relationships and the timing of domestication events for fungal cultivars have been largely inconclusive. We sequenced the genomes of 12 ambrosia beetle fungal cultivars and bark beetle associates, including the devastating laurel wilt pathogen, *Raffaelea lauricola*, to estimate a robust phylogeny of the Ophiostomatales. We find evidence for contemporaneous diversification of the beetles and their associated fungi, followed by three independent domestication events of the ambrosia fungi genus *Raffaelea*. We estimate the first domestication of an Ophiostomatales fungus occurred ~86 Ma, 25 million years earlier than prior estimates and in close agreement with the estimated age of farming in the Platypodinae (96 Ma). Comparisons of the timing of fungal domestication events with the timing of beetle radiations support the hypothesis that the first large beetle radiations may have spread domesticated “ambrosia” fungi to other fungi-associated beetle groups, perhaps facilitating the evolution of new farming lineages.

KEYWORDS

ectosymbiosis, genomics, insect agriculture, life history evolution, symbiosis

1 | INTRODUCTION

Fungal farming by insects evolved independently in at least three insect orders: Hymenoptera (ants), Blattodea (termites) and Coleoptera (beetles) (Mueller & Gerardo, 2002). The most well studied of these insect fungiculture systems evolved 61–57 million years ago (Ma) in the Attine ants (Branstetter et al., 2017) which include the leaf-cutters of Central and South America (Currie et al., 2003; De Fine Licht et al., 2013; Mueller, Rehner, & Schultz, 1998; Suen et al.,

2011). The evolution of agriculture has resulted in colony sizes numbering in the millions for leaf-cutter ants, making them the dominant herbivore in the New World Tropics (Holldobler & Wilson, 1990). Insect fungiculture also evolved with a single origin 40–25 Ma (Roberts et al., 2016) in the Macrotermitinae termites, which subsequently diversified and spread through Africa and Asia (Mueller & Gerardo, 2002; Aanen & Boomsma, 2005). The third insect–fungal agricultural symbiosis is between ambrosia beetles of the weevil family Curculionidae and (primarily) “Ophiostomatoid” fungi of the

orders Ophiostomatales and Microascales. Ambrosia beetles make up the most speciose and diverse assemblages of both insects and cultivars with an estimated 3,400 species of beetles (Farrell et al., 2001) and a probably comparable number of domesticated fungal species (de Beer & Wingfield, 2013). Within Curculionidae, agriculture evolved multiple times including a minimum of 14 independent transitions to fungal farming in the subfamily Scolytinae (Hulcr & Stelinski, 2017). In contrast, a single transition to fungal farming occurred in the subfamily Platypodinae with all but two of the ~1,400 species practicing fungiculture. The wholly ambrosial “core” Platypodinae (consisting of the tribes Tesserocerini and Platypodini) has a maximal stem age estimated at 119 Ma and a minimum crown age of 88 Ma, making them the oldest known insect farmers (Jordal, 2015).

Scolytinae is comprised of ~4,000 species of bark beetle and ~2,000 species of ambrosia beetle (Farrell et al., 2001; Jordal & Cognato, 2012), the most important distinction between the two beetle types being their primary food source. Both bark and ambrosia beetles are wood-borers that spend the majority of their adult lives under the bark or in the sapwood of dead or dying trees. Bark beetles, however, feed primarily on the plant tissue of these trees, often including the relatively nutrient-rich phloem (i.e., phloeophagy or phloeomycetophagy). A tiny minority of bark beetle species which attack and kill living trees (~1% of 6,000 Scolytines) (Kirkendall, Biedermann, & Jordal, 2014) have gained notoriety for landscape-scale forest devastation throughout North America (Raffa et al., 2008; Tsui et al., 2011). In contrast, ambrosia beetles colonize dead and dying trees by boring past the bark into the sapwood and cultivating fungal “gardens” that breakdown dead tissue and concentrate nutrients for the beetles’ consumption (i.e., xylomycetophagy or mycetophagy) (Kasson et al., 2013). “Ambrosia” is a general term used to reference the fungi farmed by ambrosia beetles, and it is well recognized that like the beetles, these fungi are multiply derived within several fungal orders (Cassar & Blackwell, 1996; de Beer & Wingfield, 2013).

Both bark and ambrosia beetles form obligate symbioses with fungi. Morphological adaptations to this lifestyle evolved in many species in the form of exoskeletal fungal “pouches” called mycangia (Francke-Grosman, 1956) which ensure faithful transmission of symbiont fungal spores. The fungi have reciprocally adapted to insect dispersal by evolving sticky spores on the tips of fruiting structures that act to facilitate attachment to their beetle vector (Malloch & Blackwell, 1993) and by fulfilling beetle nutrition by producing thickened, nutrient-rich conidia (“ambrosial growth”) (Neger, 1908). In their association with bark beetles, Ophiostomatoid fungi serve as nutritional symbionts (Bentz & Six, 2006; Six & Klepzig, 2004), terpenoid detoxifiers (Wang et al., 2012, 2014) and pheromone producers (Blomquist et al., 2010). From these mutualisms, more intimate symbioses evolved in the form of domestication and cultivation by groups of farming ambrosia beetles (Batra, 1963; Farrell et al., 2001; Jordal, Normark, & Farrell, 2000). Ambrosia fungi are fully domesticated, vertically transmitted and serve as the sole food source for their insect caretakers. While domestication results

in a loss of independence, these fungi are compensated with dispersal to new environments and protection from competing fungi.

Many fungi within the order Ophiostomatales (Ascomycota, Sordariomycetes) are involved in mutualisms with bark and ambrosia beetles. Genomic studies of these fungi have primarily focused on tree-killing bark beetle associates due to the economic impact of these forest pests during outbreaks (DiGuistini et al., 2009). At the time this study began, there were 12 published Ophiostomatales fungal genomes, nine were bark beetle associates, two were human or animal pathogens, and one was an ambrosia beetle fungal cultivar. In 2002, an invasion of the ambrosia beetle *Xyleborus glabratus* to the southeast United States caused alarm when it was recognized to be the vector of *Raffaelea lauricola*, the fungal pathogen responsible for the deadly tree disease known as “laurel wilt” (Fraedrich et al., 2008). Hundreds of millions of Redbay trees throughout the region have succumbed to the disease with other tree species in the *Lauraceae* family, including the avocado *Persea americana*, showing signs of susceptibility (Ploetz et al., 2012). This outbreak has prompted a number of studies attempting to describe the biology and mechanism of pathogenicity for *R. lauricola* (Harrington & Fraedrich, 2010; Inch & Ploetz, 2011; Ploetz et al., 2012).

Past efforts to definitively reconstruct evolutionary relationships among Ophiostomatales genera have been inconclusive. The inferred phylogenies were often poorly supported, or the relationships among taxa were unstable across studies, with results varying depending on the taxa or loci sampled, and on the phylogenetic methods employed (Bateman et al., 2017; Dreaden et al., 2014; Massoumi Alamouti, Tsui, & Breuil, 2009; Musvuugwa et al., 2015; Taerum et al., 2013). Phylogenetic uncertainty makes it difficult to address long-standing questions regarding the timing and number of domestication events for ambrosial cultivars in the Ophiostomatales (Dreaden et al., 2014). We wanted to address uncertainties regarding the taxonomy, systematics, number of domestication events and timing of these events in the beetle-associated Ophiostomatales. We used both Illumina and Pacific Biosciences technologies to sequence and annotate 11 Ophiostomatales and one Microascales genomes and transcriptomes. This data set provides the first annotated draft genome for the laurel wilt pathogen, *R. lauricola*, and doubles the number of Ophiostomatales genomes available for comparative genomics. We combined these data with existing genomes from the fungal order and performed a phylogenomic analysis with 18 ingroup taxa and four outgroups. Extensive topology hypothesis testing was performed to address any lingering uncertainty in the resulting whole-genome tree as well as to test hypotheses regarding the monophyly of *Raffaelea*. We used the resulting whole-genome topology as a constraint tree in a phylogenetic analysis with fewer loci but with expanded taxon sampling. This strategy resulted in a well-supported phylogeny for the major clades within the Ophiostomatales as well as confident ancestral state reconstruction (ASR) for life history habit. Additionally, comprehensive molecular dating analyses, bolstered by fossil evidence, were performed revealing the timing of both the origin of Ophiostomatales and multiple fungal domestication events.

2 | METHODS

2.1 | Culturing and library preparation

2.1.1 | Illumina genome sequencing

Fungal cultures were grown at room temperature in 25 ml malt extract broth (17 g malt extract/litre) for 1–5 weeks. Tubes were centrifuged at >4,500 g to remove excess liquid, decanted, and the pellet frozen and ground under liquid nitrogen. Powdered fungal extract was immediately split into several 1.5-ml tubes for nucleic acid extraction. Genomic DNA was isolated using the Mo Bio PowerSoil[®] DNA Isolation Kit (Mo Bio Laboratories Inc.)

For genome sequencing, PCR-free multiplexed sequencing libraries were created using the Illumina TruSeq kit with an average insert size of 180 bp. The libraries were pooled in equimolar ratios and sequenced using 101-bp PE sequencing on an Illumina HiSeq 2000. Mate-pair libraries were constructed using the Illumina Nextera Mate Pair kit and size selected to ~4 kb using Pippin Prep (Sage Science). Fragments were circularized and sheared to ~800 bps using a Covaris S220 sonicator. Libraries were pooled in equimolar ratios and sequenced both as 101 bp PE reads on an Illumina HiSeq 2000 and as 259 bp PE reads on an Illumina MiSeq. Quality filtering and trimming of mate-pair Illumina reads were performed using NextClip (Leggett, Clavijo, Clissold, Clark, & Caccamo, 2013).

2.1.2 | Pacific Biosciences genome sequencing

Fungal cultures were grown at room temperature in 25-ml liquid malt extract medium for 3–8 weeks. Tubes containing cultured fungal tissue were centrifuged and decanted to remove excess liquid, and then, fungal tissue was pressed between filter paper discs to further remove liquid. Samples were frozen at –80°C and then transferred to a 4.5-L Labconco benchtop freeze dryer system and dried for >4 hr. Freeze-dried samples were lyophilized using two steel beads per tube with 1–2, 20-s cycles, oscillating at 30 Hz. It is notoriously difficult to extract ultrapure DNA from Ophiostomatoid fungi. To obtain the more than 10 µg of ultrapure, high molecular weight DNA required for Pacific Biosciences (PacBio) SMRT sequencing, we modified the Qiagen Genomic Tip 500 g extraction kit protocol as follows. Lyophilized tissue was added to 12 ml of Y1 buffer containing 1,000 units of lyticase and mixed thoroughly. Each sample was incubated approximately 2 hr at 30°C before it was pelleted and resuspended in 15 ml of buffer G2 containing 30 µl of RNase A (100 mg/ml). 1 ml of Proteinase K stock solution was added, and the buffer was incubated at 50°C for 8–12 hr. Finally, the remaining cellular debris was pelleted and the supernatant retained for processing as outlined in Part II of the Genomic-tip protocol for isolation of genomic DNA. This process was repeated until ~75 µg of HMW DNA was obtained. This HMW DNA was subsequently purified using a MoBio PowerClean Pro DNA Cleanup Kit. While this step ensures ultrapure DNA for PacBio library construction, it reduced total yield to ~15 µg. The integrity and final

concentration of genomic DNA were determined using pulse-field gel electrophoresis, fluorescence and absorbance spectrophotometry.

Sequencing libraries were prepared using a SMRTbell Template Prep Kit 1.0 with BluePippin size selection for fragments >20 kb. The resulting libraries were sequenced on four single-molecule real-time (SMRT) cells using P6 version 2 chemistry and reagents by SciLife labs in Uppsala, Sweden.

2.2 | Transcriptome library preparation and sequencing

Total RNA was isolated using the Ambion Ribopure Yeast Kit (Life Technologies). RNA quality and concentration were assessed using either a Bioanalyzer 2100 with an Agilent RNA 6000 Nano Kit (Agilent Technologies) or an Agilent Tape Station 2100. Libraries were prepared from 4 µg total RNA using the Illumina TruSeq Stranded RNA Kit following the manufacturer's protocols. The resulting cDNA libraries were pooled in equimolar ratios and sequenced using an Illumina HiSeq 2500 with either 100-bp or 150-bp PE reads. Illumina reads were trimmed and quality filtered using Trimmomatic version 0.30 (Lohse et al., 2012) with the HEADCROP option enforced for RNA-seq reads.

2.3 | Genome assembly and annotation

2.3.1 | Illumina sequenced libraries

Preliminary assemblies of paired-end reads were performed with Velvet (Zerbino & Birney, 2008) and AllpathsLG (Butler et al., 2008) with similar results. Final assemblies, which included mate-pair data, were performed with AllpathsLG. RNA-seq reads were assembled using Trinity (Grabherr et al., 2011; Haas et al., 2013) with the jaccardclip option enforced for fungal genomes.

Protein annotation of draft assemblies was performed using the MAKER 2.3.36 pipeline (Altschul, Gish, Miller, Myers, & Lipman, 1990; Camacho et al., 2009; Holt & Yandell, 2011; Slater & Birney, 2005). Repetitive elements were masked by first generating classified repeat libraries for each assembled genome using RepeatModeler (Bao, 2002; Benson, 1999; Smit, Hubley, & Green, 1996; Price, Jones, & Pevzner, 2005; Smit & Hubley, 2008). This repetitive sequence library was combined with a MAKER provided transposable element library and was used in RepeatMasker 3.0 (Smit et al., 1996) for masking within the MAKER pipeline. Both protein and RNA-seq data were used as evidence for generating “hints” for ab initio gene predictors. We used the Trinity assembly and the published transcriptome from *Grosmannia clavigera* (DiGuistini et al., 2011) as transcript evidence for each species. Whole proteomes for *G. clavigera* (DiGuistini et al., 2009), *Ophiostoma piceae* (Haridas et al., 2013) and *Magnaporthe grisea* (Dean et al., 2005) were provided as protein evidence. Iterative rounds of training were used to produce hidden Markov models for the ab initio gene predictors SNAP (Korf, 2004) and Augustus (Stanke, Schöffmann, Morgenstern, & Waack, 2006) while self-training was used for GeneMark-ES (Ter-Hovhannisyanyan,

Lomsadze, Chernoff, & Borodovsky, 2008). Ab initio gene predictors are notorious for overcalling genes (Larsen & Krogh, 2003; Skovgaard, Jensen, Brunak, Ussery, & Krogh, 2001), we set MAKER to only call gene models which had RNA-seq or protein evidence ($AED < 1$) supporting them (`keep_preds = 0`). The remaining ab initio gene calls were then scanned for protein family domains (PFAM) using InterProScan5 (IPRscan) (Jones et al., 2014). Predicted proteins lacking functional evidence but that contained at least one PFAM domain were promoted to full gene models using scripts included with MAKER.

Functional annotation of the final protein sets was accomplished by first scanning each proteome using the command line version of InterProScan5. The `ipr2gff3` script bundled with MAKER was used to update the MAKER-generated `gff3` with the resulting IPRscan information. BLASTP was then used to search each protein set against the UniProt Swiss-Prot database. The best blast hit for each protein was used as the closest gene I.D. for the query protein. The “`maker_functional_fasta`” and “`maker_functional_gff`” scripts bundled with MAKER were used to update the respective FASTA and `gff3` files with BLASTP results. Finally, genome completeness was assessed using BUSCO 2.0 (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) on the annotated protein set.

2.3.2 | PacBio sequenced libraries

Genome assembly was performed using the hierarchical genome assembly process (HGAP) (Chin et al., 2013) as implemented in SMRT Portal. Genome-guided transcriptome assembly was performed using Trinity with the `jaccardclip` option enforced. Protein annotation of HGAP assemblies was performed using the MAKER3 with Evidence Modeler (Haas et al., 2008) pipeline as described above with the following modifications. Proteins from all annotated Ophiostomatales genomes, including the six proteomes annotated from Illumina assemblies in this study, were used as evidence for ab initio gene predictors. Genome-guided Trinity assemblies from all species submitted for PacBio sequencing were used as EST evidence.

At the time this analysis began, there were several Ophiostomatales genomes for which only nucleotide sequence was available. To include these genomes in our analysis, we first had to perform protein annotations using MAKER. *Ophiostoma novo-ulmi* (Forgetta et al., 2013) was annotated as described for the Illumina-sequenced genomes above using MAKER2. There has recently been a functional annotation of *O. novo-ulmi* published (Comeau et al., 2015); however, since our annotation was completed before this publication, we used our version in all downstream analyses. Functional annotation for *Graphilbum fragrans* (B. D. Wingfield et al., 2015) and *Lep-tographium procerum* (van der Nest et al., 2014) was performed with MAKER3 as described for the PacBio sequenced genomes above.

To determine the number of ribosomal operons present in our PacBio sequenced genomes, we performed iterative BLAST searches, first using rDNA sequences obtained from GenBank as the initial query. Once a full-length operon was identified for each species, it was extracted and used as a query in a second round of BLAST

searches to determine the number of partial and full-length operons present in each genome.

2.4 | Phylogenomic analysis

Phylogenomic analyses for eighteen Ophiostomatales genomes (11 from this study) and four outgroup taxa were performed. Single-copy, orthologous, protein sequences were identified using OrthoMCL (Li, 2003; van Dongen, 2000) using various inflation parameters ranging from 1.5 to 8. Ortholog sequences were aligned using the L-INSI-I algorithm in MAFFT version 7.271 (Kato & Standley, 2013) and poorly aligned regions were trimmed using GBLOCKS (Castresana, 2000; Talavera & Castresana, 2007). Final alignments were analysed using supertree and supermatrix approaches. For the supertree approach, a best fitting evolutionary model of amino acid substitution was selected for each aligned and trimmed amino acid sequence using automatic model selection with free rate heterogeneity incorporated in IQTree 1.5.3 (Nguyen, Schmidt, von Haeseler, & Minh, 2015). Maximum-likelihood (Felsenstein, 1981) phylogenies with 1000 UltraFast bootstrap replicates (Minh, Nguyen, & von Haeseler, 2013) were generated for each locus. We used CONSENSE 3.695 (Felsenstein, 2005) to build consensus bootstrap trees for each loci with all poorly supported nodes ($<95\%$ UF bootstrap support) collapsed. IC values were then calculated for an extended majority rule consensus (eMRC) tree using only the remaining, well-supported nodes with RAXML 8.2.9 (Salichos & Rokas, 2013; Salichos, Stamatakis, & Rokas, 2014).

For the supermatrix approach, the same loci used to generate the eMRC tree were concatenated into a single alignment. A maximum-likelihood phylogeny with 1,000 UltraFast (UF) bootstrap replicates was estimated using IQTree 1.5.3 with a posterior mean site frequency (PSMF) model (Wang, Minh, Susko, & Roger, 2017) and 60 mixture classes. This analysis was repeated three times to ensure convergence in topologies. IC values were calculated for the supermatrix topology using the well-supported bootstrap trees from the supertree analysis. The resulting phylogenies were viewed and manipulated using DENDROSCOPE 3.2.10 (Huson & Scornavacca, 2012) and EvoView 2 (He et al., 2016).

2.5 | Choosing among alternative phylogenetic hypotheses

The topologies obtained in the supermatrix and supertree phylogenomic analyses were very similar with the exception of the position of the root node. To differentiate among these alternatives and to compare alternative hypotheses around nodes with low support values, we used the approximately unbiased (AU) test (Shimodaira, 2002) implemented in IQTree1.5.3. Additionally, we used the AU test to compare alternative topologies regarding the monophyly of *Raffaelea* (see Fig. S1). We estimated the likelihood of 12 different topologies under the PSMF model with 60 rate classes using the concatenated amino acid data set. A parsimony starting tree was used to estimate the site frequencies for the 60 rate classes, and 10,000 RELL replicates were performed for each tested tree.

2.6 | Molecular dating

We used PhyloBayes 3.3 (Lartillot, Lepage, & Blanquart, 2009) with site-specific evolutionary rates modelled using nonparametric infinite mixtures (CAT-GTR) to generate an ultrametric tree from our whole-genome concatenated tree. A diffuse gamma prior with the mean equal to the standard deviation was used for the root of the tree. A log-normal autocorrelated relaxed clock model (Thorne, Kishino, & Painter, 1998) with a Dirichlet prior on divergence times was used for dating the tree.

We employed an alternative dating strategy to take advantage of the existing fungal fossil record outside the Ophiostomatales. First, 73 (75 including two outgroup species) whole proteomes spanning Basidiomycetes and Ascomycetes (including a subsample of available genomes in the Ophiostomatales) were clustered using OrthoMCL (Li, 2003; van Dongen, 2000). The resulting 562 single-copy orthologs present in at least 75% of species were aligned and trimmed using MAFFT version 7.271 and Gblocks as above. A maximum-likelihood phylogeny was estimated for the group using RAXML 8.2.4 with GTR+G. To date the resulting phylogeny, six different fossils spanning Basidiomycete/Ascomycete split (Dikaryons) were used as minimum constraints on nodes. Two of these were in the Basidiomycetes and correspond to a minimum age of 90 My for the Agaricales (Hibbett, Grimaldi, & Donoghue, 1997) and a minimum age of 360 My (Stubblefield, Taylor, & Beck, 1985) for the Basidiomycota in general. The remaining four fossils were all of Ascomycete origin. The oldest of these, *Paleopyrenomycites devonicus*, was used to constrain the minimum age of the Pezizomycotina to 395 My (Taylor & Berbee, 2006; Taylor, Hass, Kerp, Krings, & Hanlin, 2005). The second Ascomycete calibration was a minimum age of 136 My for the origin of the Diaporthales (Bronson, Klymiuk, Stockey, & Tomescu, 2013). The third Ascomycete calibration was a minimum age of 99 My for the origin of the genus *Ophiocordyceps* (Sung, Poinar, & Spatafora, 2008). Finally, 50 My was used as the minimum age for the origin of the genus *Aspergillus* (Dorfelt & Schmidt, 2005).

To determine how taxon sampling and fossil calibrations affected dates within the Ophiostomatales, the 75 taxon data set was pared down to 54 whole Ascomycete genomes and the four ascomycete fossil calibrations described above. The Ascomycete taxon set was independently dated using three different amino acid alignments of 60 loci (24,404 amino acids), 40 loci (12,552 amino acids) and 30 loci (11,790 amino acids). To further investigate the effects of fossil constraints and taxon sampling, the 54 taxon Ascomycete data set was pared down to 35 Sordariomycetes (38 with three outgroups) and the *Ophiocordyceps* and *Aspergillus* fossil calibrations from above. The 35 Sordariomycete taxon set was dated using the same 40 locus alignment used in the 75 Dikaryons and the 54 Ascomycete above. Finally, in order to date the 18 whole Ophiostomatales genomes (22 with four outgroup taxa), we used the 95% credibility intervals from our prior dating analyses to constrain two nodes within the Ophiostomatales. The root node was constrained to a maximum of 165 My and a minimum of 56 My. The node corresponding to the most recent common ancestor of *R. lauricola* and *G. clavigera* was

constrained to 150 and 46 My. A single fossil calibration was used in the outgroup to constrain the origin of the Diaporthales (Bronson et al., 2013) to a minimum age of 136 My (see Figure 1). The 22 taxon tree was then dated using two independent amino acid alignments of 30 loci (14,763 aa) and 40 loci (18,991 aa). For each alignment, the Markov chain was run for 15,000 steps with two independent runs to ensure convergence. 5,000 steps were discarded as burn-in with each chain sampled every 10 steps for a total of 1,000 samples used to estimate divergence times for each run.

2.7 | Phylogenetic analysis and ancestral state reconstruction

We used the resulting topology from our concatenated phylogenomic analysis as a constraint tree in a second phylogenetic analysis, which included a more comprehensive sampling of Ophiostomatales species. Where possible, we used published GenBank (Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2016) sequences for ribosomal small subunit (SSU), ribosomal large subunit (LSU), beta-tubulin (tubB), elongation factor-1 alpha (EF1A) and RNA polymerase II (RPB2) genes for many Ophiostomatales. When genomes were available, we extracted full-length sequences from the contigs therein. For species without all gene regions in GenBank, the regions that were available were used. Accession numbers for GenBank sequences are listed in Table S1. Seventy-one ingroup and 15 outgroup (86 total) taxa were included, the majority of which overlapped with taxa in Dreaden et al. (2014). To survey for appropriate outgroup taxa, rDNA sequences from several *Raffaelea* species were compared to GenBank using BLASTN (Camacho et al., 2009) while excluding Ophiostomatales. The resulting closest hits were used as outgroups as well as those species for which genomic data are available and were used in our phylogenomic analyses. Sequences were aligned using MAFFT version 7.271 (Katoh & Standley, 2013) and visually adjusted using AliView (Larsson, 2014) and Mesquite 3.2 (Liu, Endara, & Burleigh, 2015). PartitionFinder 1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012) was used to find the optimal combination of Eleven data partitions (SSU, LSU, 1st, 2nd and 3rd codon positions of each protein coding gene) resulting in eight partitions modelled with GTR+G using eight gamma rate categories (Tavaré, 1986; Yang, 1993). A maximum-likelihood phylogeny was estimated using RAXML 8.2.9 with 100 bootstrap replicates. The resulting bootstrap trees were filtered in RAXML (-J MR_DROP flag) to eliminate "rogue taxa" (Aberer, Krompass, & Stamatakis, 2013; Pattengale, Aberer, Swenson, Stamatakis, & Moret, 2011) that could mislead the resulting phylogenetic and ASR analyses. After the removal of two taxa (*Afro-raffaelea ambrosiae* CBS141678 and *Raffaelea ellipticospora*, 86 taxa topology included as Fig. S3), the maximum-likelihood analysis was repeated with 200 bootstrap replicates.

We used Mesquite to reconstruct ancestral character states for fungal farming on the 84 taxon constrained tree with three different methods: parsimony, likelihood (Pagel, 1999) and stochastic character mapping (Huelsenbeck, Nielsen, & Bollback, 2003; Nielsen, 2002). With parsimony, we reconstructed ancestral states using models that

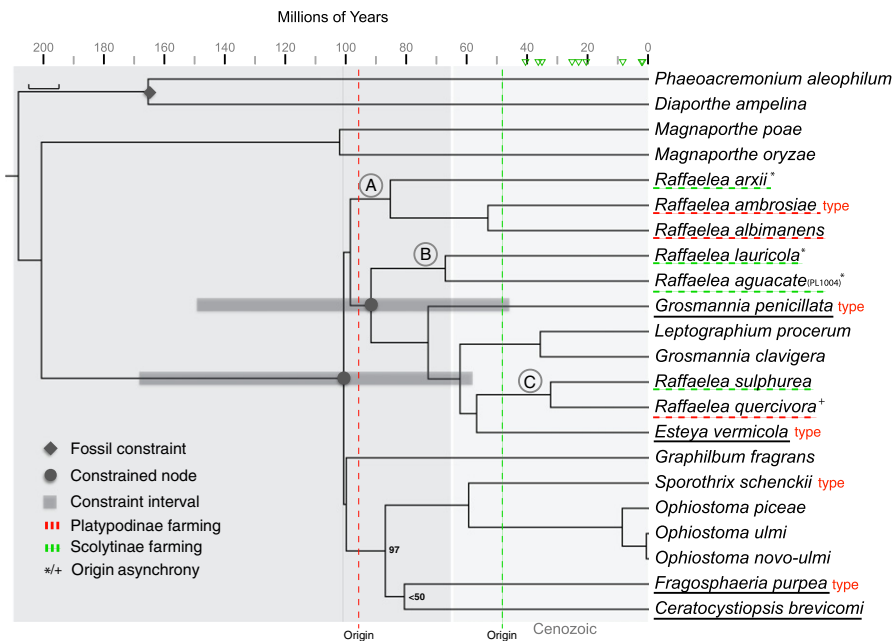


FIGURE 1 The timing of domestication events in Ophiostomatales are indicated by A, B and C. The origin of farming in Platypodinae (red dotted line) is from Jordal (2015) while the timing for the known origins in Scolytinae (green line and triangles) is from Jordal and Cognato (2012) and Gohli et al. (2017). Underlined taxa are genomes sequenced in this study, colours indicate farming beetle associate (Platypodinae/Scolytinae); however, many *Raffaelea* species are promiscuous and associate with more than one beetle. *Indicates an asynchrony in the origin of farming and domestication, that is, farming in the ancestor of the beetle associated with this fungus evolved *after* the ancestor of this fungus was domesticated. †Indicates an asynchrony in origin where the farming in the ancestor of the beetle host evolved *before* the ancestor of this fungus was domesticated. Maximum-likelihood phylogeny estimated from 978,795 aligned amino acids (2,207 single-copy, concatenated and trimmed loci) using IQTree 1.5.3 with a posterior mean site frequency model and 60 mixture classes. A single fossil constraint was implemented in the outgroup species while upper and lower bounds derived from multiple fossil constrained analyses (see Methods) were placed on the ingroup node and the crown node encompassing the *Grosmanhia/Leptographium/Esteya/Raffaelea* clade (grey bars). Underlined species represent genomes sequenced in this study. Type species for the genus are indicated in red. Nodes receiving less than maximum bootstrap support are indicated with their respective values

included unordered parsimony, 2:1 forward-biased stepmatrix and a 1:2 reversal-biased step matrix. In estimating the likelihood reconstruction of ancestral states, we used an asymmetrical Markov k-state 2-parameter model with root state frequencies equal to the equilibrium rate. Rates were optimized from the data by first estimating a single rate under the Mk1 model (Lewis, 2001) with a starting value of 1.0. Next, we estimated both a forward and reverse rate under three scenarios: symmetric forward and reverse rates with starting values of 1.0 and 1.0, a forward-biased scenario with 1.0 and 0.1 starting values and a reverse biased scenarios with 0.1 and 1.0 as starting values. Rates were optimized under each condition and the strategy resulting in the highest likelihood was used in the reconstruction (option 3 in the Mesquite optimization settings). This same model was used in the likelihood stochastic character mapping approach.

3 | RESULTS

3.1 | Ophiostomatales genomes vary twofold in size

The results from our sequencing effort for both Illumina and PacBio technologies are summarized in Tables 1, 2 and S2. The PacBio assemblies produced significantly higher median N50 values with significantly fewer scaffolds than the Illumina assemblies (*t* test

$p = .0006$, $p = .024$, respectively). Genome assembly sizes (Table 1) varied within the Ophiostomatales by as much as twofold; however, assembly sizes are often lower than actual genome sizes, especially for short-read assemblies due to the collapsing of repeat regions. Nevertheless, the number of annotated genes correlates with assembly size regardless of the sequencing technology used (Fig. S2). BUSCO (Simão et al., 2015) statistics of genome completeness generated using 290 fungal-specific BUSCOs were not different (*t* test $p = .44$) regardless of sequencing technology used (Table 1). This is an indication that the evidence-based annotation pipeline used here is robust across a range of genome assembly qualities.

For each PacBio assembly, there were multiple large contigs that appear to be whole chromosomes with telomere repeat sequences at the start and end of the contig. We used the number of telomere repeat sequences, as well as contig size and coverage (to ensure repeats were not spurious) to estimate the number of chromosomes for each PacBio sequenced species (Table 2). Additionally, we used BLASTN coupled with coverage to identify the mitochondrial genome in these same species (Table 2).

Genome annotation produced a broad range in the number of annotated proteins within the Ophiostomatales (Table 1). We annotated previously sequenced (but not annotated) genomes from *Graphilbum fragrans* (8,878 protein coding genes) (Wingfield et al., 2015),

TABLE 1 Assembly and annotation statistics for 12 fungal genomes sequenced this study

Species	N50 (mb)	Assembly size (mb)	Max scaffold (mb)	Number scaffolds	Per cent complete ^a	Filtered gene models
<i>Ambrosiella xylebori</i>	1.86	27.1	4.32	38	94.1%	6,503
<i>Ceratocystiopsis brevicomi</i>	1.5	20.6	3.49	198	95.2%	6,327
<i>Raffaelea ambrosiae</i>	3.53	40.7	6.22	69	100%	9,913
<i>Raffaelea arxii</i>	2.2	36.7	5.56	123	98.9%	10,816
<i>Raffaelea lauricola</i>	2.87	34.6	4.91	206	98.2%	9,553
<i>Raffaelea aguacate</i>	0.46	35.9	2.39	414	97.9%	10,194
<i>Raffaelea sulphurea</i>	0.55	23.8	2.47	157	99.6%	7,774
<i>Esteya vermicola</i> ^b	4.98	33.97	7.11	29	96.9%	8,012
<i>Fragosphaeria purpea</i>	5.1	34.75	8.11	25	99.0%	8,493
<i>Grosmannia penicillata</i>	3.49	27.68	4.75	43	99.3%	7,284
<i>Raffaelea albimanens</i>	5.27	39.04	6.77	11	97.9%	9,715
<i>Raffaelea quercivora</i>	3.69	26.41	5.2	23	99.3%	8,003

Grey shading indicates species sequenced with PacBio, all other sequenced with Illumina short insert libraries and scaffolded with mate-pair libraries.

^aDenotes fraction of 290 complete fungal BUSCOs present in annotated protein set (does not include fragments).

^bDenotes estimated statistics for this species after filtering for contaminant.

L. procerum (8,430 protein coding genes) (van der Nest et al., 2014) and *O. novo-ulmi* (8,314 protein coding genes) (Forgetta et al., 2013). Strain information and accession numbers for the corresponding sequencing projects are given for each annotated genome in Table S3. Protein and transcript FASTA files as well as gff3 files containing functional annotation are available at Dryad <https://doi.org/10.5061/dryad.tk569>.

3.2 | A resolved phylogeny for the Ophiostomatales

After alignment, trimming and concatenation of 2,207 single-copy orthologs present in 18 Ophiostomatales genomes and four outgroup taxa, our concatenated alignment was 978,795 amino acids long. The use of whole-genome protein alignments to resolve the Ophiostomatales phylogeny resulted in slightly different topologies for the supertree and supermatrix approaches. In the supermatrix topology, estimated under a more complex substitution model (Figure 1), the tree is rooted on the internal branch separating *Graphilbum*, *Sporothrix schenckii*, *Fragosphaeria purpea*, *Ceratocystiopsis brevicomi* and *Ophiostoma* genera from the *Raffaelea*, *Grosmannia*, *Esteya vermicola* and *Lep-tographium* genera. *Graphilbum* (Gf), *Fragosphaeria* (Fp) and *Ceratocystiopsis* (Cb) are all genera represented by a single taxon in this analysis resulting in long terminal branches. Additionally, the available outgroup genomes are comprised of distantly related taxa. The topological differences between the supertree (Fig. S1) and supermatrix (Figure 1) analyses are characterized by whether the long terminal branches of Gf, Fp and Cb associate with the long outgroup branches. The supertree estimated using the relatively simple models available in the automatic model tests implemented in IQTree 1.5.3, roots the tree on the long branch leading to Gf (Fig. S1), indicating a potential long-branch attraction issue (Felsenstein, 1978).

The supermatrix topology had maximal bootstrap support for all but two nodes. However, it is well established that concatenation will

often result in artificially inflated support values that mask uncertainty in the data. Internode certainty values (IC) estimated using topologies from the 2,207 supertree loci indicate conflict among individual loci with respect to the supermatrix topology (data not shown). We collapsed poorly supported nodes (<95% UF bootstrap support) in the 2,207 loci trees used to calculate IC values for the supermatrix tree. While this strategy improved average IC values for the tree, low IC values still persisted for several nodes (data not shown). Low IC values are indicative of either different evolutionary histories among loci or incorrectly resolved trees due to low or noisy phylogenetic signal (Sali-chos & Rokas, 2013). However, in this case, differences in the position of the root between the supertree and supermatrix were driven entirely by six of the 2,207 topologies recovered from our supertree analysis. Additionally, both supertree and supermatrix topologies split the ambrosial genus *Raffaelea* into three distinct clades, making the genus polyphyletic. We used the AU test to explore the differences between the supertree and supermatrix topologies, evaluate nodes that were uncertain and to test hypotheses of *Raffaelea* monophyly. The 12 topologies compared in the AU test and their associated likelihood values can be seen in Fig. S1. The supermatrix topology was significantly better than the other topologies tested. IQTree 1.5.3 automatically runs a suite of topology tests in the course of performing the AU test, and the supermatrix tree was preferred among all topologies tested here. Results for all tests are reported in Table S4.

3.3 | Ancient origins and multiple domestication events within the Ophiostomatales

Results of the molecular dating analysis can be seen in Figure 1 and Table 3. The order Ophiostomatales originated ~101 Ma during a rapid radiation that gave rise to two major groups, the *Ophiostoma*/*Sporothrix*/*Fragosphaeria*/*Ceratocystiopsis* group and the *Grosmannia*/*Lep-tographium* group.

TABLE 2 Ribosomal operon counts and estimated number of chromosomes identified for genomes sequenced with PacBio

Species	Complete operons	Partial operons	Average ^b % identity (SD)	Telomere repeats	Estimate the number of chromosomes	mtGenome size (kb)
<i>Esteya vermicola</i> ^a	14	3	99.29 (0.30)	12	6	NA ^a
<i>Fragosphaeria purpea</i>	24	4	99.52 (0.81)	10	5	58
<i>Grosmanina penicillata</i>	33	9	99.60 (0.52)	16	8	200
<i>Raffaelea albimanens</i>	9	1	99.91 (0.05)	12	6	139
<i>Raffaelea quercivora</i>	29	2	99.79 (0.28)	12	6	152

Operons were considered full length if the BLASTN match was ~80% of the full-length query sequence. Mitochondrial scaffolds were determined using BLAST coupled with coverage statistics (all identified contigs had ~20× higher coverage).

^aIndicates low-level contaminant present in the sequenced culture. Only scaffolds that could be unambiguously identified were used in the final analyses; therefore, this count may be an underestimate of operon number.

^bIndicates average identity to a single query sequence from the same species.

Raffaelea/Leptographium/Esteya group. The ambrosial genus *Raffaelea* appears to have at least three different origins, the earliest of which arose ~86 Ma (crown node age, Figure 1A, Table 3). This clade contains the type species for the genus, *Raffaelea ambrosiae*, as well as *Raffaelea albimanens* and *Raffaelea arxii* (henceforth RA clade). The second origin of ambrosia fungi occurred ~67 Ma, corresponding to the crown node for the split of *R. lauricola* + *Raffaelea aguacate* (referred to as the *R. lauricola* complex in prior studies, henceforth referred to as the RL clade) (Figure 1B, Table 3). The final origin of fungal domestication within the Ophiostomatales occurred ~33 Ma (Figure 1C, Table 3) when the clade containing *Raffaelea sulphurea* and *Raffaelea quercivora* split from the lineage that became the nematophagous species *Esteya vermicola*.

The results of the 2,207 loci supertree analysis show similar support for a three domestications hypothesis. Short single-copy ortholog sequences are unlikely to accurately resolve very deep divergences so we collapsed all nodes that were not strongly supported (<95% UFbootstrap) in each of the 2,207 maximum-likelihood trees. There were 117 loci that strongly supported the *Leptographium/Grosmanina/Esteya/Rsulphurea* clade (LGER) grouping with the next most frequent conflicting topology (16) supporting a *R. sulphurea* + *Leptographium/*

Grosmanina sister group relationship. Twenty-nine loci strongly support RL+LGER as sister to one another with the next most frequent topology (6) being a RA + RL sister group relationship.

Once rogue taxa were filtered from the ingroup, 69 taxa remained in our phylogenetic analysis. The resulting phylogenetic tree had a well-resolved backbone with bootstrap support >70% for the majority of nodes (Figure 2). Our use of a constraint tree resulted in strong support for the root of the tree on the internal branch between the *Ophiostoma* et al. clade and the *Raffaelea* et al. clade. This is highly relevant because the primary shortcoming of all previous analyses of the group was their inability to resolve backbone nodes with any degree of confidence.

Ancestral state reconstruction for fungal domestication events largely resulted in the same conclusion for each methodology employed. Unordered parsimony reconstructed 2–3 domestication events in five steps (not including the *Ambrosiella* sp. outgroups) with the ancestral state at the base of the RA and RL clades being equivocal. Weighted parsimony with a 2:1 backward bias reconstructs only two domestication events, with the most parsimonious reconstruction requiring nine steps. Weighted parsimony with a 2:1 forward bias resulted in three unambiguous domestication events, requiring only five steps. Maximum-likelihood reconstructed three domestication events, although two were not statistically significant. The asymmetric two-parameter model resulted in the best log likelihood of -22.75451379 marginal probability reconstruction. Transition rates were asymmetric with a twofold faster forward rate of 0.3482 and a backward rate of 0.1719. Proportional likelihoods at the ancestral node leading to RA were noncultivar fungi = 0.8050, cultivar = 0.1950. For the ancestral node leading to RL noncultivar fungi = 0.8041, cultivar = 0.1959. For the ancestral node leading to *R. sulphurea* noncultivar fungi = 0.9490, cultivar = 0.051. The majority of stochastic mapping realizations resulted in the tree seen in Figure 2, which is in agreement with both the likelihood reconstruction and the two most parsimonious ASRs.

TABLE 3 Dates for major splits within the Ophiostomatales

Node	Mean minimum (crown) age	Mean maximum (stem) age
Ophiostomatales origin	101 ± 25	199 ± 49
<i>R. arxii</i> + <i>R. ambrosiae</i> + <i>R. albimanens</i>	86 ± 22	99 ± 25
(<i>R. laur</i> + <i>R. aguacate</i>) + (<i>Gros.</i> + <i>Lept.</i> + <i>Esteya</i> + <i>Raff</i>)	92 ± 23	99 ± 25
<i>R. lauricola</i> + <i>R. aguacate</i>	67 ± 17	92 ± 23
<i>Esteya</i> + <i>R. sulphurea</i> + <i>R. quercivora</i>	58 ± 15	63 ± 17
<i>R. sulphurea</i> + <i>R. quercivora</i>	33 ± 10	58 ± 15
(<i>Fragosphaeria</i> + <i>Cerato</i>) + (<i>Ophiostoma</i> + <i>Sporothrix</i>)	88 ± 22	100 ± 25

Three origins of ambrosia fungi (grey rows). The minimum age for the split of *R. arxii* + *R. ambrosiae* + *R. albimanens* is older than any prior estimate for the evolution of the farming habit in any insect and corresponds closely with the origin of Platypodinae.

4 | DISCUSSION

4.1 | *Raffaelea* monophyly and fungal domestication

Previous studies have disagreed about whether the ambrosia fungi genus *Raffaelea* is monophyletic (Farrell et al., 2001; Harrington,

Aghayeva, & Fraedrich, 2010; Massoumi Alamouti et al., 2009; Plötz, Hulcr, Wingfield, & de Beer, 2013) or polyphyletic (de Beer & Wingfield, 2013; Dreaden et al., 2014; Musvuugwa et al., 2015; Simmons et al., 2016). The rapid radiation at the base of the Ophiostomatales, compounded by limited data, made it impossible for these studies to confidently resolve deep nodes among major clades within the group. This makes conclusions regarding ASR, taxonomic classification and comparative genomics as robust as the underlying phylogeny on which they are based. In the studies where *Raffaelea* is polyphyletic, the clade containing *R. sulphurea* (henceforth referred to as RS) is more closely related to the *Leptographium/Grosmania* species. Our phylogenomic analysis indicates that the RL clade shares a more recent common ancestor with the LGER group than with the RA clade which contains the *Raffaelea* type species, *R. ambrosiae* (Figure 1). Furthermore, the crown node for RA is ~20 million years older than that of RL, although the RL stem node slightly overlaps with the RA crown node (Table 3). Maximum-likelihood ASR indicates there were three independent domestication events among taxa currently assigned to *Raffaelea*. de Beer and Wingfield (2013) suggested the RL complex might not share a most recent common ancestor with *Raffaelea sensu stricto* and stated: "...their generic status and position in the Ophiostomatales should await further investigation, including more gene regions, before any new combinations are made."-pg. 34. With the inclusion of ~2,200 more gene regions, the analysis presented here fulfils these requirements and validates their viewpoint. Heeding this advice, we elected to exclude *Afroraffaelea ambrosiae* from our primary conclusions on domestication as there was no support for its correct position within the Ophiostomatales (similar to when the species was first described (Bateman et al., 2017)). It is possible that the rapid radiation at the base of the Ophiostomatales resulted in a mis-rooting in our supermatrix analysis, one that would unite the RA and RL clades with increased taxon sampling and a different data set. While we cannot completely exclude this possibility, the RL+LGER relationship was recovered in our 75 whole-genome Dikarya analysis (562 loci), our 54 whole-genome Ascomycete analysis (293 loci), and our 36 whole-genome Sordariomycete analysis (375 loci) (data not shown). Additionally, a three domestications hypothesis was supported in our supertree analysis and in our topology tests (see Results above). It is worth noting, that to facilitate primer design, included in the online supplementary of this publication is ~6,500 bp of aligned CDS from 22 fungal species spanning >200 My of evolution. Newly collected CDS data can be aligned to this matrix and coupled with the phylogenomic constraint tree (Figure 1) for quick placement of additional taxa. The alignment and the Newick formatted constraint tree from Figure 1 are available on Dryad (<https://doi.org/10.5061/dryad.tk569>).

The challenges associated with accurate ASR are well established (Ekman, Andersen, & Wedin, 2008; Goldberg & Igić, 2008; Griffith et al., 2015; Litsios & Salamin, 2012; Wright, Lyons, Brandley, & Hillis, 2015). Issues such as phylogenetic uncertainty, taxon sampling and transition rates of a character state can influence the reconstruction. To ameliorate these issues as much as possible, we took

three different approaches. First, we expanded taxon sampling to include a total of 69 ingroup taxa and 15 outgroup taxa. Increased taxon sampling has been demonstrated to improve phylogenetic accuracy (Hillis, 1998; Philippe et al., 2011; Zwickl & Hillis, 2002) which in turn can increase the accuracy of ASR. Second, we used the supermatrix topology from our phylogenomic analysis as a constraint tree in our phylogenetic analysis. This serves to stabilize previously unresolved backbone nodes in the tree as well as the position of the root. Third, we reconstructed ancestral character states using three different methodologies (following the suggestion of Ekman et al., 2008). Maximum-likelihood, unordered parsimony, and forward-biased, weighted parsimony all reconstructed three domestication events for species currently in *Raffaelea*. Only one possible reversal from farming back to bark or phloem feeding has ever been detected in ambrosia beetles (Hulcr & Stelinski, 2017). Similarly in the ambrosia fungi, there have been no detected cases, of which we are aware, of cultivars reverting to a free-living state. Due to the fungi's dependence on the beetle for protection from competitors and desiccation, transportation to new substrates, as well as very limited sexual reproduction in the fungi (Musvuugwa et al., 2015), reversals seem unlikely (Farrell et al., 2001). Our models still allowed reversals, which makes our estimates conservative. More realistic models would make reversals very unlikely or exclude this possibility altogether, likely resulting in the reconstruction of even more origins of domesticated cultivars.

4.2 | PacBio sequencing produces chromosome-length scaffolds in small fungal genomes

A useful aspect of this study is the direct comparison of different genome sequencing approaches applied to multiple, small eukaryotic genomes of similar size, gene density and repeat content. While slightly more expensive per base, PacBio sequencing of small fungal genomes is the best approach in several respects. First, the hands-on time required for generating large amounts of high molecular weight DNA required for PacBio library preparation was far less than the time invested for building multiple PE and mate-pair libraries. Additionally, as the PacBio sequencing was performed on four SMRT cells for each genome, the average coverage we generated was higher than necessary for accurate genome assembly, and one could get by with less. All together, this suggests that PacBio genomes produce a lower cost per genome compared with Illumina sequencing that includes mate-paired library construction and sequencing, at least for typical Sordariomycete genomes.

One of the issues associated with genome assembly using Illumina short reads is their difficulty in spanning and resolving large repetitive regions. Our short-read assembled genomes produced no assembled ribosomal operons because of this limitation. We were able to overcome this to some extent by reassembling these genomes using Velvet (Zerbino & Birney, 2008) with a low coverage cut-off of ~300x. This resulted in assemblies for the mitochondrial genome and a ribosomal operon for most species; however, this approach does not produce ordered and orientated ribosomal

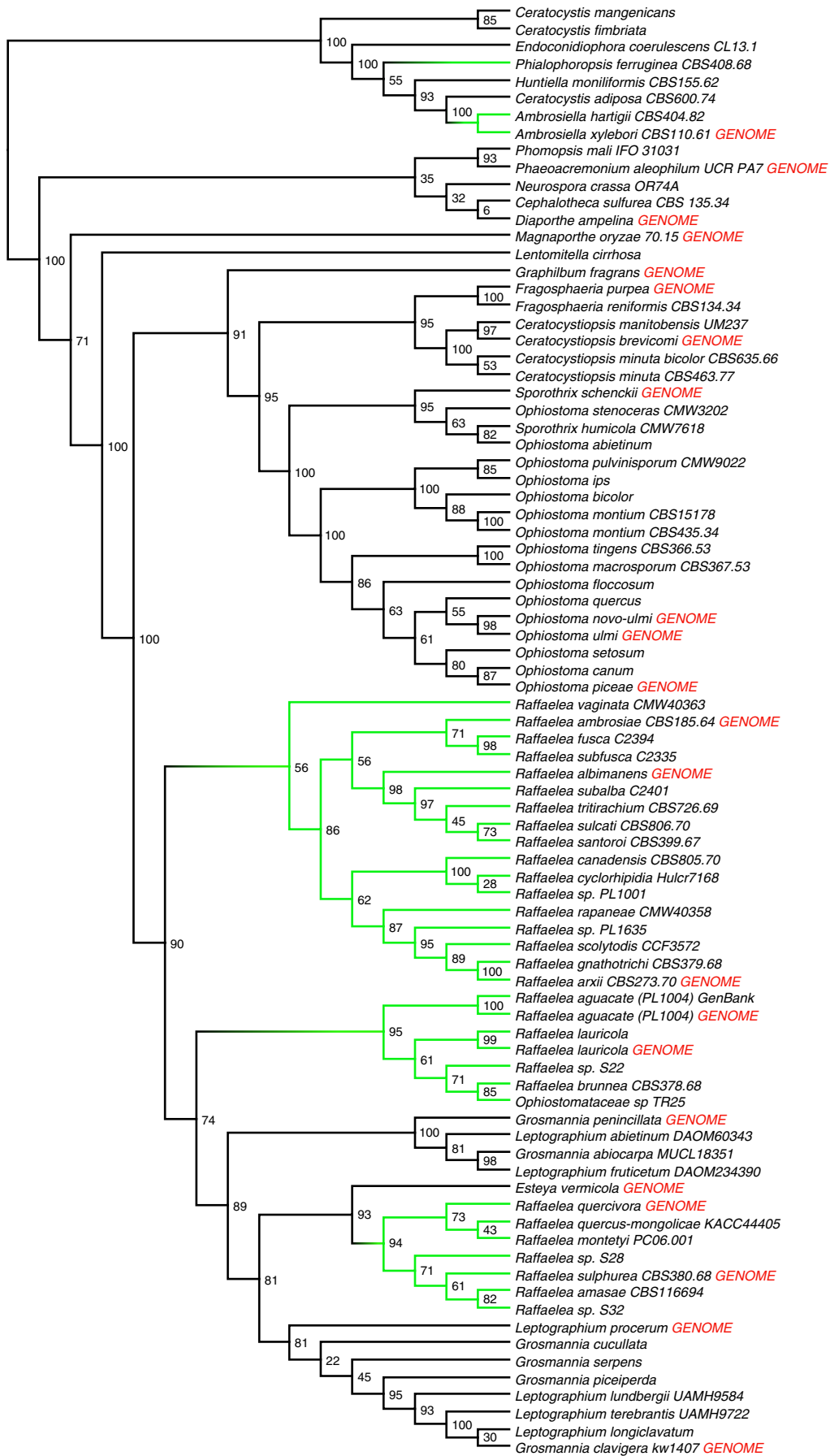


FIGURE 2 Maximum-likelihood phylogeny estimated for 84 taxa with many of the available Ophiostomatales sequences from GenBank (see Table S1) including SSU, LSU, β -tubulin, EF1- α , rpb2 (when available). RAxML 8.2.9 was used to estimate the tree using eight separate data partitions modelled using GTR+G and the whole-genome tree in Figure 1 as a constraint tree. Stochastic character mapping for the origin of domestication (green) was based on marginal probability reconstruction with an asymmetric two-parameter model with both rates estimated. Forward rate: 0.34821476, reverse rate: 0.17193707, $-\log$ Likelihood: 22.75451379

operons in a given genome, nor does it produce an accurate operon count. In contrast, our PacBio assemblies allowed us to ascertain both the placement within a scaffold and accurate copy numbers for most ribosomal operons (Table 2).

4.3 | Fungal farming begets fungal farming

With an origin in the mid-cretaceous (119–88 Ma), the Platypodinae is the oldest known group of fungal farming insects (Jordal, 2015; Jordal & Cognato, 2012; McKenna, Sequeira, Marvaldi, & Farrell, 2009). Their origin and initial diversification coincides with the diversification of other advanced weevil groups in the Curculionidae, including the bark and ambrosia beetles of Scolytinae. McKenna et al. (2009) hypothesize that the massive diversification of Curculionidae during this time was driven by the evolution and diversification of the “core” eudicots, a group comprising ~70% of all angiosperms (Friis, Pedersen, & Crane, 2016). Lacking representatives of all nonfarming Platypodine beetles, Jordal (2015) was unable to precisely date the origin of farming behaviour in the Platypodinae. However, one can infer it occurred with the emergence of the wholly farming “core” Platypodinae consisting primarily of two tribes, the Tesserocerini and Platypodini. In this study, we demonstrate that the beetle-associated order Ophiostomatales arose ~101 Ma, a time that coincides with the initial period of diversification in Platypodinae and Scolytinae weevils (Jordal, 2015; Jordal & Cognato, 2012; Jordal, Sequeira, & Cognato, 2011; McKenna et al., 2009). The short internode branches at the base of the Ophiostomatales tree (Figure 1) are indicative of a rapid radiation, resulting in two primary fungal groups, one containing the genera *Ophiostoma*/*Sporothrix*/*Fragosphaeria*/*Ceratocystiopsis*/*Graphilbum* and the other containing the genera *Grosmannia*/*Raffaelea*/*Leptographium*/*Esteya*. Closely following this initial radiation, ~86 Ma (crown node), the first fungal cultivars arose with the clade containing the *Raffaelea* type species *R. ambrosiae* + *R. arxii* and *R. albimanens* (Figure 1A, Table 3). While slightly younger, the crown node age is similar to both the diversification of the core eudicots and Platypodinae. Additionally, for sparsely sampled clades such as this, it is unlikely the oldest lineages representative of that clade will be sampled, resulting in consistent underestimate for the age of the crown node. This same bias is likely to be true for the estimated ages of the Platypodinae (Jordal, 2015) and the Scolytinae (Gohli et al., 2017) though less pronounced as estimates for both were based on more extensive taxon sampling. This bias makes it likely that the actual age of the first fungal cultivars is slightly older than 86 million years making them contemporaries of Tesserocerini, the first farming beetle tribe which arose 96 Ma (Figure 3, 95% credibility 88.5–103.4) (Jordal, 2015).

It seems reasonably certain that the Tesserocerini domesticated the first fungi ~86 Ma, because fungal farming did not evolve in the Scolytinae (and the Platypodini did not yet exist) until ~48–60 Ma (Figure 3) (Farrell et al., 2001; Gohli et al., 2017; Jordal, 2015; Jordal & Cognato, 2012). The reason for this lag in the diversification of farming beetle lineages has been puzzled over since it first became apparent (Jordal, 2015; Jordal & Cognato, 2012; Jordal et al., 2011; McKenna et al., 2009). Jordal (2015) speculated that unfavourable conditions for the fungi contributed to low diversification rates and that when temperatures increased ~60 Ma with the onset of the Paleocene-Eocene temperature maximum (PETM) (Zachos, Pagani, Sloan, Thomas, & Billups, 2001), a corresponding increase in farming lineages is observed. Additionally, Jordal points out that favourable conditions during this time likely contributed to the diversification of other taxonomic groups, as well as the evolution of agriculture in ants. While increased global temperature could have played a part in widespread taxonomic diversification, Jordal cautions that other factors such as ecological release brought on by the K-T boundary extinction event (Labandeira, Johnson, & Wilf, 2002) could not be dismissed.

Another possible explanation is that the presence of fungal cultivars facilitates the evolution of the farming habit in the beetles. In addition to inhabiting the same environment and substrate as ambrosia beetles, bark beetles maintain facultative and obligate nutritional symbioses with fungi (Klepzig & Six, 2004). The introduction of ambrosia fungi to their environment would make it a relatively short evolutionary step to fungal farming behaviour. This idea was proposed as a potential model of transition to insect agriculture between beetles and ants (Sanchez-Pena, 2005), but was dismissed as unlikely due to inaccurate (at the time) dating of farming in the beetles and fungal domestication events (Mueller, Gerardo, Aanen, Six, & Schultz, 2005). While it is still unlikely to have occurred between beetles and ants, this model is more parsimonious for some of the independent origins of farming within ambrosia beetles. Several models have been proposed for the transition to agriculture in insects (reviewed in Mueller et al., 2005) but the initial transition to farming in the Tesserocerini likely occurred via a “transmission first” model (Mueller, Schultz, Currie, & Adams, 2001). In this scenario fungi associated with beetle host plants adapt to become dispersed by the beetle. Over time beetles would become dependent on these fungi through nutrient supplementation during passive feeding (much like what is observed in bark beetles today). Eventually cultivation evolves via behavioural/genetic elements that promote increased fungal production capable of sustaining large groups, for example, burrowing, tending, cropping (Biedermann, Klepzig, & Taborsky, 2009; Biedermann & Rohlf, 2017). After evolving a farming habit

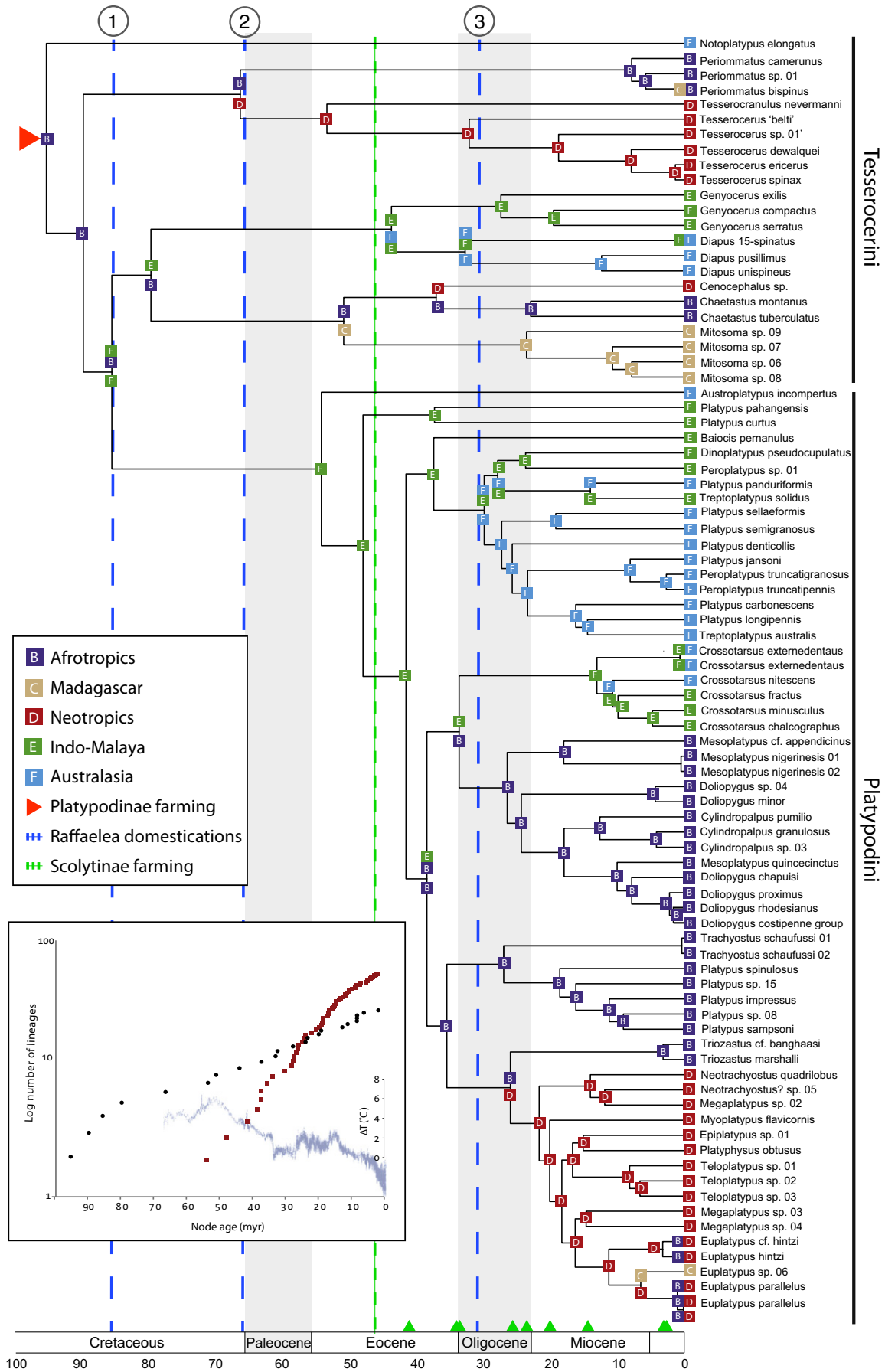


FIGURE 3 A dated phylogeny estimated from five genes for the Platypodinae ambrosia beetles. Three domestication events of ambrosia fungi currently in *Raffaelea* are labelled 1, 2 and 3 (blue lines). The earliest origin of farming in Scolytinae is indicated by the green dotted line, with subsequent times of origin indicated with green triangles. The timing for the origins of farming behaviour in Scolytine beetles is from Jordal and Cognato (2012) and Gohli et al. (2017). All known instances of farming in Scolytinae evolved after the radiation of Platypodinae. This figure is modified with permission from B.H. Jordal. Inset: lineage-through-time plots for Tesserocerini (black dots) and Platypodini (red squares), with Zachos et al.'s (2001) reconstruction of global average temperature changes plotted in blue. Ancestral areas reconstructed based on the dispersal–extinction–cladogenesis model with dispersal probabilities modelled according to Morley (2003) and Clayton, Soltis, & Soltis, (2009) (see Jordal, 2015 for details)

~96 Ma, the Tesserocerini maintained a relatively modest diversification rate with only 1–2 lineages existing outside of their point of origin in the Indo-Malaya and Afrotropics (Jordal, 2015). Perhaps brought on by the onset of the PETM (Figure 3 inset) ~55 Ma the Tesserocerini, and especially the Platypodini, experienced massive diversification events, radiating throughout Australasia, Madagascar and the Neotropics (Figure 3). This radiation would have likely permeated the existing tropical forests of the time, exposing many fungus-associated bark beetles to fully domesticated fungal lineages. The difficulty of simultaneously evolving a farming habit and domesticating a fungal lineage would have been circumvented, explaining why shortly after the initial radiation 4 of the 10 farming lineages (for which timing of origin is known) in Scolytinae appeared (Jordal, 2015; Jordal & Cognato, 2012) (see Figure 3). Additionally, while increased temperatures during the PETM may have served as the impetus for beetle radiations and facilitated fungal colonization, fewer than half of Scolytine farming lineages arose near the end of this period and the remaining arose after temperatures cooled to near pre-PETM values (Figure 3 inset, and see Jordal & Cognato, 2012 and Gohli et al., 2017 for details on timing). By comparison, farming in ants and termites has evolved only once despite their undoubtedly intimate contact with various fungi in their environment. In stark contrast to ambrosia beetles (excluding *Austroplatypus* and perhaps some *Ambrosiophilus* species) (Kasson et al., 2016), farming ants and termites are eusocial and form large, well-defended, colonies to the exclusion of other insect species. This social structure likely impedes the comingling of cultivars and behaviour in a common substrate with other nonfarming, mycophagous species (Mueller et al., 2005). This requires nonfarmers to domesticate fungi “from scratch” in order for a new instance to arise.

If the presence of fungal cultivars enables nonfarming bark beetles to transition to fungal farming, we might expect to see evidence of this in beetle-fungal association patterns observed today. We would expect to see an increase in the rate of evolution of beetle farming lineages with a corresponding increase (perhaps with a time lag) in the number of domesticated fungal lineages. An increase in the rate of evolution of beetle farmers is clear: in the first 50 million years of fungal farming, 1 lineage evolved. In the 50 My following PETM radiations, at least 14 lineages evolved. As existing farmers domesticated new fungal lineages (Kasson et al., 2013, 2016; Mayers et al., 2015), an asynchrony between the age of the beetle's farming habit and the time of domestication for fungal lineages is observed. An example of this asynchrony is the Scolytine genus *Gnathotrichus* which did not evolve a farming habit until 16–34 Ma (95% credibility

crown node, Gohli et al., 2017) despite its association with *R. gnathotrichi*, a very close relative of *R. arxii*, a lineage whose ancestor was domesticated ~86 Ma (Figure 1A). The reciprocal scenario is observed in the association of *Platypus quercivorus* and *R. quercivora*, where farming evolved in the *Platypus* ancestor ~96 Ma, but the *R. quercivora* ancestor was domesticated just ~33 Ma (Figure 1C, Table 3). A similar situation appears to have occurred in the Microascales cultivars where existing farming lineages appear to have independently domesticated at least three new fungal lineages (Mayers et al., 2015). Studies to confirm the exact nature of beetle-fungi domestications are confounded by the fact that beetle-fungi fidelity is (usually) limited, and beetles of the same species can carry multiple or different symbionts (Batra, 1966; Biedermann, Klepzig, Taborsky, & Six, 2013; Harrington et al., 2010; Kostovcik et al., 2014). It is possible there were two domesticated fungal lineages when the Platypodinae radiated. The timing for the origin of the first cultivar coincides with the origin of the farming tribe Tesserocerini ~86 Ma and includes the ancestor of the type species *R. ambrosiae*. The second fungal cultivar to emerge ~67 Ma was the ancestor to RL, and it appears this timing largely overlaps with very long stem subtending the Platypodini radiation (Figure 3). Whether the ancestor to RL was domesticated by the Platypodini prior to their radiation is difficult to ascertain given the beetles' propensity for cultivar switching and cosmopolitan distribution. Further interrogation of this question will require exhaustive sampling and dating of all domesticated fungal lineages from four Ascomycete orders (and 3 known Basidiomycete species) (Kolarik & Kirkendall, 2010; Li et al., 2015). Comparisons of cultivar age to dated phylogenies of the Platypodinae and Scolytinae farmers (Gohli et al., 2017; Jordal, 2015; Jordal & Cognato, 2012) are required to determine whether (i) the farming habit evolved first (fungi newly domesticated), (ii) The cultivar arose first (beetle recently evolved farming habit) or (iii) both farming and cultivar arose contemporaneously (farming evolved “from scratch”).

5 | CONCLUSIONS

Coincidence in the timing and diversification of Curculionidae and Ophiostomatales fungi suggest codiversification, perhaps driven by the radiation of the “core” eudicots. It is possible these fungi initially inhabited the same substrate as the beetle and were transient hitchhikers during beetle dispersal. Eventually this association evolved into a more intimate symbiosis similar to what we see today among bark beetles and fungi. Shortly after the initial radiation ~96 Ma, the

ancestor to Platypodinae ambrosia beetles made an evolutionary advance when it domesticated a fungus to serve as its sole food source. Despite a robust symbiosis with fungi related to Ophiostomatales cultivars, it would be another 50 million years before the sister group to Platypodinae, Scolytinae, evolved a farming habit. We propose that this lag could potentially be caused by the difficulty of simultaneously evolving a farming behaviour and domesticating a fungal lineage with characteristics adequate to serve as a dependable, sole food source. It is clear that after the Platypodinae radiations occurred they were quickly followed by five independent Scolytinae transitions to the farming habit with five more (that have been dated) occurring over the next ~35 million years. Additionally, with the increased number of farming beetle lineages, we expect an increased rate of new fungal lineage domestications. Without knowing the timing of all domesticated fungal lineages, it is impossible to say for certain whether the positive feedback loop suggested here actually occurred.

Doubtless there are ambrosia beetles other than Platypodinae in which farming evolved “from scratch” in the past 100 million years. This study highlights the importance of having a well-resolved, dated phylogeny for both the beetle (Gohli et al., 2017; Jordal, 2015) and their fungal cultivars for determining whether this is the exception rather than the rule for the evolution of new farming lineages. Future studies that include dating the origins of all fungal cultivars in both Ascomycete and Basidiomycete lineages will help clarify how the evolution of farming lineages proceeds.

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DATA ACCESSIBILITY

Genome/transcriptome sequencing and assembly project BioProject: PRJNA395605; GenBank accessions PCDD00000000–PCDD00000000; NCBI SRA Sample accessions: SAMN07414043–SAMN07414054.

The resulting annotation files including gff3 file, protein FASTA files and transcript FASTA files for all taxa annotated this study are available at Dryad <https://doi.org/10.5061/dryad.tk569>.

Supermatrix alignment, supertree alignments, 86 taxon five gene alignment with defined data partitions file available at: Dryad <https://doi.org/10.5061/dryad.tk569>.

AUTHOR CONTRIBUTIONS

D.V. and J.M. conceived the study. D.V. cultured, extracted and constructed PE and MP libraries for all species except for *C. brevicomi*. R.B. cultured, extracted and constructed PE libraries for *C. brevicomi*. D.V. performed bioinformatic and phylogenetic analyses. D.V. wrote the manuscript with contributions from J.M. and R.B. All authors read and approved the final manuscript.

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