Genome diversification of symbiotic fungi in beetle-fungus mutualistic symbiosis

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Abstract

Ambrosia beetles and their fungal symbionts represent a widespread and diverse insect-fungus mutualism. This study investigates the genomic adaptations associated with the evolution of the ambrosia lifestyle across multiple fungal lineages. We performed comparative genomic analyses on 70 fungal genomes from four families (Irpicaceae, Ceratocystidaceae, Nectriaceae, and Ophiostomataceae), including 24 ambrosia and 34 non-ambrosia lineages. Our phylogenomic analyses reveal multiple independent colonization of insect vectors by the fungi, spanning from the mid-Cretaceous (114.6 Ma) to the early Quaternary (1.9 Ma). Contrary to expectations for obligate symbionts, ambrosia fungi showed no significant genome-wide reductions in size, gene count, or secreted protein repertoire compared to their non-symbiotic relatives. Instead, we observed complex, lineage-specific patterns of genomic adaptation. Key findings include lineage-specific expansions in carbohydrate-active enzyme families (AA4 in Nectriaceae, CE4 in Ophiostomataceae, and GH3 in Ophiostomataceae and Ceratocystidaceae), suggesting potential enhancement or loss of lignin modification, hemicellulose deacetylation, and cellulose degradation in different ambrosia lineages. Repeat-Induced Point mutation analysis revealed family-specific patterns rather than lifestyle-associated differences. These results highlight the diverse genomic strategies employed by ambrosia fungi, demonstrating that symbiont evolution can proceed

through refined, lineage-specific changes rather than genome-wide, or convergent alterations. Our study suggests that unlike other insect-farmed fungi, ambrosia fungi are not domesticated crops, but rather free-living fungi which co-opted wood boring beetles as vectors via subtle, lineage-specific adaptations.

Introduction

Insect-fungal symbioses represent remarkably stable and successful evolutionary strategies with significant ecological impacts. Insects and fungi engage interactions ranging from mutualistic to commensal to competitive, and are pivotal in nutrient cycling and decomposition of wood. Among these relationships, the ambrosia symbiosis is the most widespread, diverse, and repeatedly evolved, It involves the wood-boring ambrosia beetles in the weevil subfamilies Scolytinae and Platypodinae, and their fungal partners from several fungal orders. Ambrosia beetles vector fungi between dead trees - the fungal substrate - and feed on fungal conidia within the tunnels. The fungi benefit from beetle-assisted transport to new tree hosts. This mutualism shows co-evolution, with beetles developing mycangia to carry fungal spores (Francke-Grosmann, 1963; Hulcr & Stelinski, 2017), and fungi adapting ambrosial cells to enhance nutrient provisioning (Freeman et al., 2013; Harrington et al., 2014; Huang et al., 2020). Both partners have specialized adaptations supporting their interdependence.

Both the ambrosia beetles and the ambrosia fungi exemplify convergent evolution, with multiple independent origins across different lineages (Hulcr & Stelinski, 2017; Johnson et al., 2018; Kirkendall et al., 2015). Beetles have 12-16 hypothesized origins of fungus farming (Johnson et al., 2018; Pistone et al., 2018), while ambrosia fungi have arisen from multiple origins in both Basidiomycota and Ascomycota (Li et al., 2015; Massoumi Alamouti et al., 2009). This convergence provides an opportunity to study genomic adaptations supporting mutualistic interactions across diverse evolutionary backgrounds.

Recent genomic studies on various fungal symbionts, including mycorrhizal fungi and fungal crops of insects, have revealed insights into genome evolution and functional gene diversification (Fu et al., 2020; Miyauchi et al., 2020; Nygaard et al., 2016; Poulsen et al., 2014). However, most such symbioses evolved only once. Ambrosia fungi and their multiple evolutionary origins offer unparalleled designs for comparative phylogenetic analyses.

Despite the appearance of uniformity of the ambrosia lifestyle, recent studies show that ambrosia fungi's metabolomic profiles don't converge across lineages (Huang et al., 2019, 2020). This lack of metabolomic convergence, combined with a strong influence of ancestral metabolic features, suggest the uniqueness of each ambrosial system, and beckons redefining what ambrosia fungi are. Ambrosia fungi are not internal symbionts, restricted to an environment controlled by their animal partner. Instead, they spend most of their life cycle outside of the animal symbiont, occupying their ancestral niche in freshly dead trees. Thus, compared to the genomes of their free-living relatives, the genomes of ambrosia fungi are unlikely to exhibit major differences in metabolism, catabolism, or perhaps even secondary compounds. Just as their relatives, ambrosia fungi are poor degraders of lignocellulose (Skelton et al., 2019).

Their key adaptations facilitate the colonization of the beetle's transport receptacle, the mycangium, and the capacity to sequester nutrients necessary for the beetle development. There is currently no robust theory predicting what metabolic pathway would be up- or down-regulated to achieve these. The adaptations might be subtle (Veselská et al., 2019). They may also be varied across different fungal lineages due to their multiple independent origins and subsequent specificity to their beetle lineages (there are few evolutionary transitions between ambrosia symbiotic systems, Hulcr and Stelinski, 2017).

Functional genes, particularly those encoding carbohydrate-active enzymes (CAZymes), lipases, peptidases, and cell wall-degrading enzymes, are crucial indicators of fungi's metabolic capabilities and ecological roles (Hernández-Martínez et al., 2016; Resl et al., 2022). However, recent research on ambrosia fungi suggests that their enzymatic profiles may be adapted differently than previously thought. Rather than being efficient at plant biomass degradation, these fungi appear to be specialized for rapid utilization of simple nutrients available in their beetle galleries (Skelton et al., 2020; Veselská et al., 2019). This challenges earlier assumptions about the role of biomass degradation in the success of the ambrosia symbiosis. Therefore, key questions remain about the nature and direction of specialization in the enzymatic profiles of ambrosia fungi, and how these profiles compare to those of their non-symbiotic relatives.

G-protein coupled receptors (GPCRs) are crucial for fungi to sense and respond to environmental cues, detecting signals such as nutrients, pheromones, and stress factors. These transmembrane proteins regulate fungal development, metabolism, and pathogenicity (Brown et al. 2018; Xue et al. 2008). In pathogenic fungi, certain GPCR families, like Pth11-like receptors, are key for host recognition and infection (Kou et al. 2017; Shang et al. 2021). However, the role and evolution of GPCRs in symbiotic fungi, particularly in ambrosia symbiosis, remain largely unexplored. Understanding the GPCR repertoire in ambrosia fungi could reveal how these symbionts perceive and interact with their beetle hosts. This knowledge shed light on the differences between ambrosia fungi and their free-living relatives or pathogenic fungi.

Candidate secreted effector proteins represent another potentially critical component of ambrosia fungal genomes. These specialized proteins are likely instrumental in mediating host interactions, particularly in the establishment and maintenance of the symbiotic relationship with their hosts (Garcia & Ané, 2016; Martin et al., 2016; Plett et al., 2011). By analogy with other fungal symbioses, such as mycorrhizal associations, these effectors may facilitate nutrient exchange, modulate host immune responses, and enable colonization of the beetle gallery ecosystem. Investigating the presence, diversity, and function of these proteins in ambrosia fungi could provide valuable insights into the molecular mechanisms underlying this intricate insect-fungal partnership.

Repeat-Induced Point mutation (RIP) is another crucial aspect of fungal genome evolution that warrants investigation in ambrosia fungi. This genome defense mechanism, which mutates and silences repetitive DNA sequences, can significantly influence genome architecture and evolution. While RIP is typically associated with sexual reproduction, ambrosia fungi are thought to be primarily asexual (Farrell et al., 2001; Harrington, 2005; Harrington et al., 2010; Normark et al., 2003). However, sporadic discoveries of sexual states in some ambrosia lineages (Mayers et al., 2017, 2020) suggest the potential for RIP

activity through cryptic sex. Examining RIP in ambrosia fungi could provide insights into their genome stability and evolution in the context of symbiosis, revealing how these fungi balance genome defense and adaptability in their specialized ecological niche (van Wyk et al., 2019; Vogel & Moran, 2013).

Our study explores the genomic foundations underlying ambrosia fungi's adaptation to their specialized ecological niche. We compared 70 fungal genomes across four families (Irpicaceae, Ceratocystidaceae, Nectriaceae, and Ophiostomataceae), including 24 ambrosia and 34 non-ambrosia lineages, plus 12 outgroups. This dataset, comprising 8 newly sequenced and 62 retrieved genomes, represents independent origins of ambrosia fungi. Our research aims to uncover distinctive genomic characteristics associated with the ambrosia lifestyle by addressing five key questions: 1) How do the genomic features of ambrosia fungi compare to those of other fungal symbionts and their non-symbiotic relatives? 2) What specific enzymatic adaptations correlate with the origins of the symbiotic relationship with ambrosia beetles? 3) How do secreted proteins and GPCRs contribute to mutualistic recognition in the ambrosia symbiosis? 4) What role does Repeat-Induced Point mutation (RIP) play in shaping the genome architecture of ambrosia fungi? This study explores the evolutionary patterns, genomic foundations, and ecological adaptations of ambrosia fungi, enriching our understanding of insect-fungal symbiosis.

Results and Discussion

Genome Assembly and Quality Assessment

We sequenced genomes of eight species in the Ophiostomataceae, Ceratocystidaceae, Nectriaceae, and Irpicaceae families, including both ambrosia and non-ambrosia fungi. Genomic features are summarized in Table S2. All genomes showed Benchmarking Universal Single-Copy Orthologs (BUSCO) values above 96%, indicating high-quality assemblies (Figure 1). Genome sizes varied across families, ranging from 28 Mb (*Ambrosiella roeperi* T.C. Harr. & McNew) to 54.4 Mb [*Neocosmospora euwallaceae* (S. Freeman, Z. Mendel, T. Aoki & O'Donnell) Sand.-Den., L. Lombard & Crous)]. GC content was relatively consistent [45% in A. roeperi to 55.3% in *Harringtonia lauricola* (T.C. Harr., Fraedrich & Aghayeva) Z.W. de Beer & M. Procter)]. Assembly contiguity varied considerably, with N50 values ranging from 0.38 Mb [(*Irpex subulatus* (Ryvarden) Z.B. Liu & Y.C. Dai)] to 4.45 Mb (*N. euwallaceae*). Scaffold counts ranged from 39 [(*Leptographium procerum* (W.B. Kendr.) M.J. Wingf., Trans)] to 476 (*I. subulatus*). Protein-coding gene counts showed substantial variation, from 8,158 [(*Huntiella moniliformis* (Hedgc.) Z.W. de Beer, T.A. Duong & M.J. Wingf.)] to 21,224 (*N. euwallaceae*). Notably, Nectriaceae family members [(*N. euwallaceae* and *N. solani* (Mart.) L. Lombard & Crous)] exhibited higher gene counts compared to other families.

No consistent genomic patterns (Figure 2 & Table 2) were observed between ambrosia and non-ambrosia fungi across families, implying that the ambrosia lifestyle may not be associated with convergent genome-wide characteristics.

Multiple symbiosis events of ambrosia fungi across Dikarya

Our phylogenomic analyses, based on genome-wide sequence data from 70 taxa—including 24 ambrosia fungi, 34 non-ambrosia relatives, two Mucoromycota outgroups (not shown in the time-calibrated phylogeny), and ten taxa from the Eurotiomycetes, Leotiomycetes, and Sordariomycetes groups—enhanced the resolution of the phylogenetic topology. The resulting phylogenomic tree indicates that ambrosia fungi colonized the insect vectors repeatedly, over a wide evolutionary period, spanning from the mid-Cretaceous (114.6 Ma) to the early Quaternary (1.9 Ma) (Table 1). This tree reveals multiple independent beetle colonization events and subsequent co-diversification (Figure 1).

Ancient Origins and Asynchronous Evolution in Ophiostomataceae

The phylogenetic topology of Ophiostomataceae in our study largely aligns with previous findings, albeit with some discrepancies. Notably, our placement of *Graphilbum fragrans* (Math.-Käärik) Z.W. de Beer, Seifert & M.J. Wingf. as the basal lineage within the Ophiostomataceae supports the findings of Nel et al. (Nel et al., 2021) and Vanderpool et al. (Vanderpool et al., 2018). This differs from the topology presented in de Beer et al. (2022), where *G. fragrans* is nested with *Leptographium*, *Dryadomyces*, and *Esteya* species. Consistent with earlier studies, our phylogeny indicates *Raffaelea* sensu stricto species clustered with a clade comprising *Leptographium*, *Dryadomyces*, *Harringtonia*, *Esteya*, and *Grosmannia* species, albeit with moderate support. While our analysis yielded 100% bootstrap support for this grouping, it is important to note that concatenated supermatrix datasets can sometimes artificially inflate support values, potentially masking underlying uncertainties (Degnan & Rosenberg, 2009; Kolaczkowski & Thornton, 2004). Gene and site concordance factors (gCF/sCF) for this node (6.68/34 respectively) closely mirror those reported by de Beer et al. (2022) (10.1/34.6). Our incomplete lineage sorting (ILS) tests (Table S3) revealed significant values for both gCF ($p = 3.85E-04$) and sCF ($p = 4.27E-07$), suggesting that introgression or incomplete taxon sampling may have influenced this branching pattern. Future studies incorporating more closely-related species are needed to provide better resolution of their evolutionary relationships. Consistent with previous research, our phylogeny also shows a well-supported clade comprising *Ophiostoma*, *Sporothrix*, *Fragosphaeria*, and *Ceratocystiopsis*.

The Ophiostomataceae exhibit variable degrees of adaptation to beetles, characterized by high levels of promiscuity in beetle host associations (Li et al., 2018; Simmons et al., 2016). Our latest estimates suggest earlier divergence times compared to previous studies, pushing the origin of the first ambrosia symbioses deeper into the past.

The *Dryadomyces* clade (corresponding to the *Raffaelea sulphurea* complex or RS clade in Vanderpool et al., 2018) diverged approximately 66.4 million years ago (Ma) (95% CI: 44.1–100.1 Ma), with a stem age of 102 Ma. This is considerably earlier than the 33 Ma crown age and 58 Ma stem age previously reported. The *Harringtonia* clade (equivalent to the *Raffaelea lauricola* complex or RL clade) shows an even older divergence at 84.7 Ma (95% CI: 60.2-119.1 Ma), surpassing the previous estimate of 67 Ma for its crown age. Notably, the *Raffaelea* sensu stricto clade (corresponding to the *Raffaelea ambrosiae* complex or RA clade) exhibits the earliest divergence at 114.6 Ma (95% CI: 86.4–152 Ma), with a stem age of 121.3 Ma. This substantially predates the 86 Ma crown age previously reported. It also

aligns closely with estimates of the origin of Platypodinae (Coleoptera, Curculionidae), the oldest clade of ambrosia beetles, at ~96 Ma by Jordal (2015), but it predates the estimate by (Shin et al., 2018).

These deep divergence times, particularly in the *Raffaelea* clade, suggest that the lineages leading to these ambrosia fungi were already distinct around or before the estimated origin of fungus-feeding in some beetle groups. While beetle mycangia morphology has been suggested to influence symbiont fidelity (Li et al., 2018; Mayers et al., 2022; Nakashima, 1975), the frequent colonization of different ambrosia beetle lineages by Ophistomataceae may reflect aspects of fungal ecology rather than beetle morphology (Hulcr & Skelton, 2023). Some fungal traits that are beneficial or conducive to transmission within the ambrosia symbiosis may have evolved prior to their association with beetles, predisposing these fungi to readily establish symbiotic relationships (Six, 2012). This is consistent with the observation that different beetle lineages may have been independently colonized by various Ophiostomataceae fungi at different times, leading to the observed pattern of asynchrony . This revised evolutionary scenario provides a more nuanced understanding of the complex and ancient nature of these symbiotic relationships, emphasizing the importance of fungal pre-adaptations in directing the ambrosia symbiosis.

Specialized Symbioses and Evolutionary Synchronization in Ceratocystidaceae

In contrast to the promiscuous nature of Ophiostomataceae, Ceratocystidaceae fungi exhibit higher fidelity to their beetle vectors. The *Ambrosiella-Meredithiella* clade diverged 34.6 Ma (95% HPD: 22.9–52.1 Ma), with crown ages closely approximating the origins of their respective hosts (Xyleborini and *Corthylus*) (Gohli et al., 2017).

The Ceratocystidaceae is a promising model for comparative evolutionary analysis of the symbiosis evolution. Several genera of ambrosia fungi evolved independently in this clade, and all are highly specific to their beetle vectors (unlike many of the Ophistomataceae) (Mayers et al., 2022; Skelton et al., 2019) (Francke-Grosmann, 1967; Mayers et al., 2020). The fungus-beetle evolutionary units differ slightly in their relationships to host trees. Species within the *Ambrosiella*/*Xylosandrus* clade are polyphagous in terms of the tree host taxonomic identity, which contributes to their success as invasive species in many parts of the world (Galko et al., 2018; Greco & Wright, 2015; Storer et al., 2017). They are, however, highly specific to a particular stage of the tree tissue death and decay. This ecological niche—freshly dead or live but internally compromised tree branches—conforms to that of the necrotrophic phytopathogenic relatives of *Ambrosiella*, and it seems to have driven the adaptation of the beetle's semiochemical ecology and host-selection behavior (Ranger et al., 2021). The *Meredithiella* lineage, associated with various Corthylini beetles, shows similarly high mutual fidelity. However, *Meredithiella* fungi appear to be more specialized in terms of tree host range, as many species are found in specific hardwood tree species (Mayers et al., 2018). Testing the extent to which these joint ecologies are driven by the fungal or the beetle ancestral ecology will require a more comprehensive sample of phylogenetic relatives of both the fungi and the beetles. Genomic data for *Wolfgangiella*, *Phialophoropsis*, and *Toshionella* remain unavailable.

The contrasting host specificity between Ophiostomataceae and Ceratocystidaceae highlight the diversity of evolutionary strategies in ambrosia symbioses, with Ophiostomataceae

retaining a more flexible, promiscuous relationship to both beetles and trees, while Ceratocystidaceae showing specialized ecologies.

Adaptability and Promiscuity in Nectriaceae Ambrosia Fungi

In Nectriaceae, the *Neocosmospora* ambrosia clade (henceforth NAC, formerly known as the Ambrosia *Fusarium* Clade or AFC) presents a unique case of fungal adaptation to the ambrosia lifestyle. This monophyletic clade diverged approximately 9.4 Ma (95% HPD: 5.8–15.1 Ma), aligning closely with the previous estimation (~19.3 Ma; 95% HPD: 10.7–28.2 Ma) in O'Donnell et al. (2015) and the divergence of their primary hosts, the *Euwallacea* beetles (~ 12 Ma) (Gohli et al., 2017). This near-synchronous divergence suggests a co-evolutionary relationship, yet not as strict as observed in some other ambrosia symbioses.

Like *Ambrosiella*, the NAC fungi are specific to the stage of tree death, but promiscuous in terms of tree taxonomy. The few studied species are parasites on stressed but living trees, or colonizers of moribund tissues, but rarely they colonize wood in advanced stages of death and decay (Carrillo et al., 2019; Kasson et al., 2013; O'Donnell et al., 2015; Wang et al., 2021). They are able to associate with multiple beetle vectors colonizing the same type of tree tissue. For instance, in Taiwan, NAC species such as AF-18 were found to be associated with three different *Euwallacea* species [Polyphagous Shot Hole Borer (PSHB), Tea Shot Hole Borer (TSHB), and Kuroshio Shot Hole Borer (KSHB)], while individual beetle species like PSHB were found to vector multiple NAC species (AF-13 to AF-18, and *N. kuroshium*) (Carrillo et al., 2019). Initial reports of strict associations in invaded areas, such as *N. euwallaceae* with *E. fornicatus* and *N. kuroshium* with *E. kuroshio*, may not represent the final specificity, but possibly the founder effect of limited initial populations.

The capacity to colonize partially living trees appears to be a native ecology of some, but not all, *Neocosmospora*/*Euwallacea* pairs (Hulcr et al., 2017). These species have become significant invasive pests worldwide, causing substantial damage to both native and cultivated trees (Liao et al., 2023; Mendel et al., 2021; M Smith et al., 2019). For example, *E. fornicatus* in California and Israel can attack over 200 woody plant species from 58 families, with about 32 species serving as reproductive hosts (Freeman et al., 2013). The damage by these invasions may be partly due to the ability of their NAC symbionts to colonize and thrive in diverse tree species, although the tree death is attributable to the slow accumulation of attacks rather than pathogenicity (Freeman et al., 2013).

Recent Divergence of Irpicaceae Ambrosia Fungi

A unique case is presented by *I. subulatus* (Irpicaceae, formerly known as *Flavodon subulatus* or *Flavodon ambrosius*), which diverged approximately 1.9 Ma (95% HPD: 1.6-54.2 Ma) according to phylogenomic analysis. This divergence time significantly postdates the estimated origin of its associated beetle hosts in the genera *Ambrosiodmus* and *Ambrosiophilus*, which evolved around 19 Ma (Gohli et al., 2017). This temporal discrepancy suggests a complex evolutionary history, potentially involving host switching or delayed specialization of the fungus to the ambrosia beetle lifestyle. It may also reflect the fact that basidiomycete ambrosia fungi are poorly sampled and virtually unstudied, even though some are associated with the most basal clades of scolytine beetles (Jusino, Skelton, Johnson, et al., 2020).

Notably, *I. subulatus* can produce free-living fruiting bodies (Jusino, Skelton, Chen, et al., 2020), a trait analogous to *Termitomyces* fungi in termite-fungus farming systems (Aanen et al., 2002). This capacity for environmental existence outside of the insect gallery, also observed in other ambrosia fungi like *R. lauricola* (Inch et al., 2012), likely represents a retained ancestral trait from its wood-inhabiting predecessors. Given the more recent divergence of *I. subulatus* compared to its hosts, this trait retention may have facilitated its transition into the ambrosia symbiosis. The ability to produce fruiting bodies confers evolutionary advantages, allowing *I. subulatus* to survive without its beetle hosts and colonize new niches. Such trait retention potentially influences the fungus's population genetics, dispersal capabilities, and evolutionary trajectory, contributing to its success across multiple beetle host species and geographic regions (Gomez & Hulcr, 2021). The apparent evolutionary mismatch between *I. subulatus* and its hosts presents an intriguing scenario for studying the dynamics of symbiont acquisition and co-evolution in ambrosia systems, particularly in cases where the symbiont may have been acquired more recently in evolutionary time. Additional ecological, biological, and genomic data on *Irpex* ambrosia fungi from their native range would greatly enhance our understanding of their evolutionary trajectories.

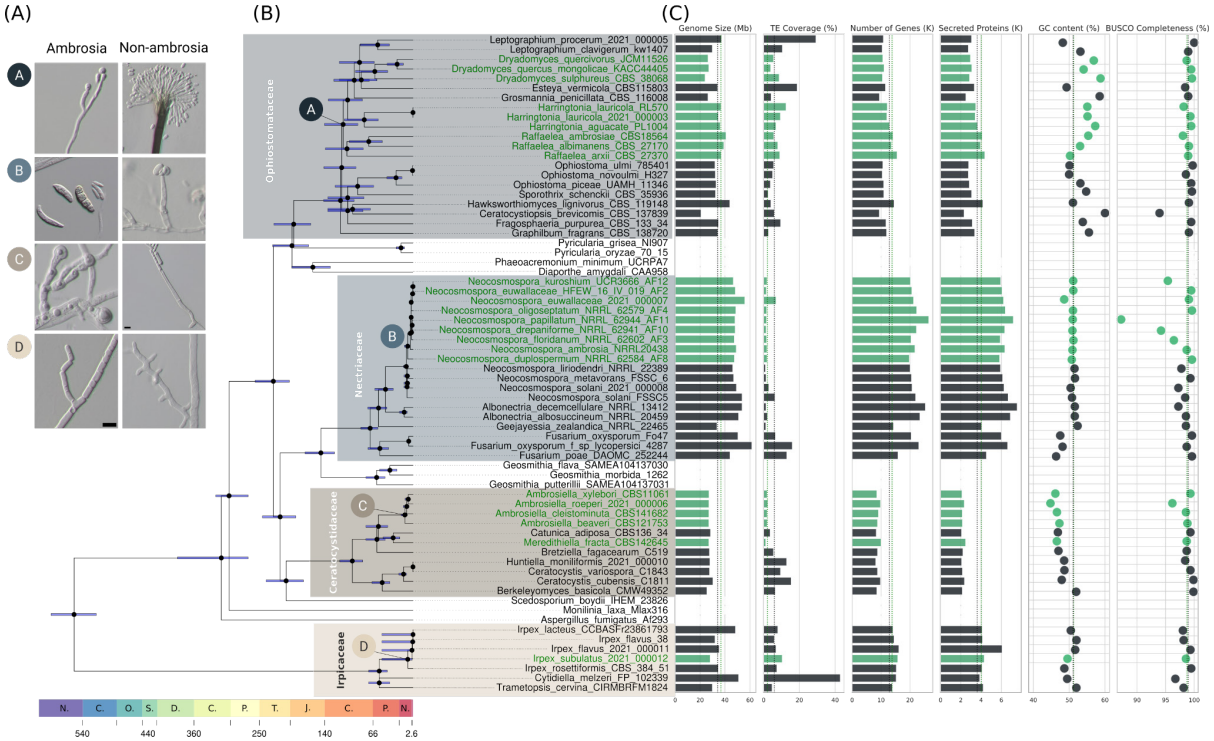


Figure 1. Evolution and genomic features of ambrosia fungi. (A) Representative fungal species illustrate morphological adaptations (enlarged cells) in ambrosia vs. non-ambrosia fungi; colored circles denote representative species in four fungal families. (B) Dated phylogenomic tree showing multiple origins of ambrosia fungi across Ascomycota and Basidiomycota. Green taxa represent ambrosia fungi. Time scale in millions of years ago (Ma) shown at the bottom. (C) Comparison of key genomic features (genome size, TE coverage, gene count, and secreted protein count) between ambrosia (green) and non-ambrosia (black) fungi. Outgroup species not shown.

Genome Features of Ambrosia Fungi Show Family-Specific Differences Without Broad Phylogenetic Trends

Our Phylogenetically Independent Contrasts (PIC) analysis revealed no statistically significant associations between the ambrosia lifestyle and the examined genomic features when accounting for phylogenetic relatedness (Figure 2 & Table 2). The analysis included genome size, transposable element (TE) coverage, number of genes, number of secreted proteins, and GC content. None of these features showed a significant difference between ambrosia and non-ambrosia fungi (Table 2, all p-values > 0.05). These results suggest that, contrary to expectations derived from studies of other symbiotic microorganisms, there are no clear, consistent genomic signatures associated with the ambrosia lifestyle across different fungal lineages. The absence of universal genomic signatures across all ambrosia fungi suggests that any adaptations to this lifestyle might be lineage-specific rather than following a general pattern. This aligns with observations in other symbiotic systems, where genomic changes often reflect the unique evolutionary trajectories and ecological contexts of specific lineages (Kohler et al., 2015; Smith et al., 2022; Tian et al., 2012). In ambrosia fungi, as in other symbiotic systems, these adaptations may be shaped by factors such as the host tree species, competing microorganisms, and specific environmental conditions (Hulcr et al., 2012; Ibarra-Juarez et al., 2018; Kostovcik et al., 2015). This pattern aligns with emerging views in symbiont evolution, where genomic changes often reflect the unique evolutionary trajectories and ecological contexts of specific lineages, rather than following a universal trend (Kohler et al., 2015; Meseguer et al., 2017).

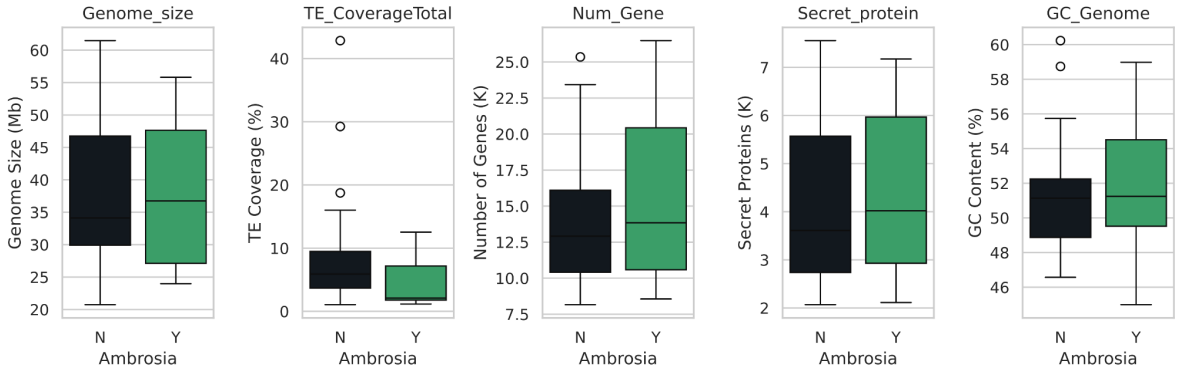


Figure 2. Comparison of genomic features between ambrosia (green) and non-ambrosia (black) fungi. No statistically significant differences were found for genome size, transposable element coverage, gene count, secreted protein count, or GC content across all species tested.

Metabolic Versatility and Genomic Stability in Ambrosia Fungi

Our analysis of functional gene families in ambrosia fungi revealed a more straightforward evolutionary picture than previously thought. Unlike the extreme genome reduction often seen in many prokaryotic symbionts (Nakabachi et al., 2006; van Ham et al., 2003) or some eukaryotic symbionts (Martin et al., 2010; Song et al., 2022), we observed nearly equivalent mean copy numbers of genes in ambrosia fungi (3.51) compared to non-ambrosia fungi (3.52). This maintenance of gene copy numbers aligns with observations in other eukaryotic

symbionts, including some mycorrhizal fungi (Martin et al., 2010; Miyauchi et al., 2020) and lichen mycobiont (Armaleo et al., 2019).

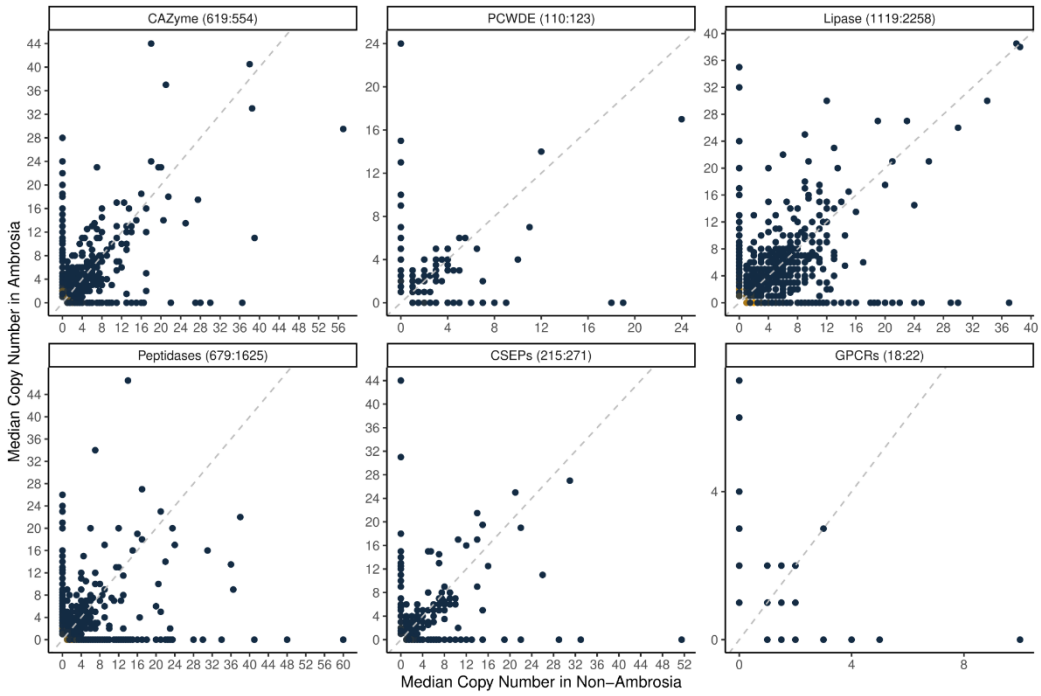
Specific gene families crucial for fungal metabolism differed between fungal clades (Figure 3A). CAZymes showed a slight increase in ambrosia fungi, with a gain/loss ratio of 1.12, while Plant Cell Wall Degrading Enzymes (PCWDEs) showed near-balance to slight reductions (gain/loss ratio of 0.89). This suggests adaptation favoring simple carbohydrate utilization over complex wood polymer degradation in ambrosia fungi compared to their free-living relatives. These findings indicate a refinement of these enzyme families rather than substantial decreases, contrasting with the loss of many biosynthetic pathways often seen in other symbionts (Kohler et al., 2015; Latorre & Manzano-Marín, 2017; Song et al., 2022).The near-balance to slight reduction in GPCRs (gain/loss ratio of 0.818) observed in ambrosia fungi likely reflects their dual nature as symbionts that retain many genetic and metabolic characteristics of their free-living relatives. Despite their symbiotic lifestyle within beetle galleries, these fungi still require mechanisms to sense and respond to environmental cues, much like their non-symbiotic counterparts. The absence of Pth11-like GPCRs, often associated with host-pathogen interactions, further suggests that ambrosia fungi have maintained environmental sensing capabilities while adapting to their symbiotic niche, rather than evolving more specialized symbiotic or pathogenic traits. Lipases and peptidases showed more widespread reductions, with gain/loss ratios of 0.495 and 0.418, respectively. However, these losses primarily involve genes with low copy numbers (Table S4). This pattern is reminiscent of gene family contractions observed in some eukaryotic symbionts, such as the yeast-like symbiont (YLS) of aphids, which maintains most metabolic pathways but shows some gene family reductions (Vogel & Moran, 2013). The observed gene reduction may be an adaptive trait, a consequence of the ambrosia lifestyle evolution, or a result of genetic drift in small, clonal populations with limited sexual reproduction. Distinguishing between these possibilities remains challenging.

A test of correlation between functional gene richness and diversity, expressed as Shannon entropy (Figure 3B) revealed a much greater influence of phylogenetic relatedness than the effect of ambrosial lifestyle CAZymes was the only functional gene tested that showed significant difference ($p = 0.006$) in relation to lifestyle, indicating a potential reduction of the ambrosia habit on carbohydrate metabolism. For other gene categories, including PCWDEs, lipases, peptidases, CSEPs, GPCRs, the lifestyle did not significantly influence functional diversity ($p > 0.05$). Gene richness data also revealed interesting patterns, with a consistently strong phylogenetic effect across all categories and a more variable impact of lifestyle. This variability in functional diversity aligns with the observation that eukaryotic symbionts often show more variable patterns of genome modification compared to prokaryotic symbionts (González-Pech et al., 2019; Nygaard et al., 2016; van de Peppel et al., 2021; Vogel & Moran, 2013). The complex interplay between phylogenetic background and lifestyle in shaping gene repertoires highlights the intricate evolutionary processes at work in these fungal lineages.

The retention of genome size and metabolic diversity in ambrosia fungi likely relates to their dynamic lifestyle. Unlike stable obligate symbionts, ambrosia fungi face constant cycles of colonization and competition in ephemeral beetle galleries. This requires maintaining diverse metabolic capabilities (Huang et al., 2019, 2020) to quickly establish dominance, outcompete other microbes (Kajimura & Hijii, 1992), and utilize varied resources. Moreover, the need to

re-enter the mycangia of dispersing adult beetles adds another layer of selective pressure (Mayers et al., 2022; Skelton et al., 2019). Ambrosia fungi must not only dominate the gallery environment but also maintain their ability to grow in a form suitable for mycangial transport (Francke-Grosmann 1967). The genomic “refinement” of ambrosia fungi can be viewed as an adaptive strategy balancing metabolic versatility with symbiotic specialization. This evolutionary trajectory allows them to remain competitive while meeting symbiotic demands, demonstrating that obligate symbiosis doesn't always lead to extreme genome reduction, especially in variable environments.

(A)



(B)

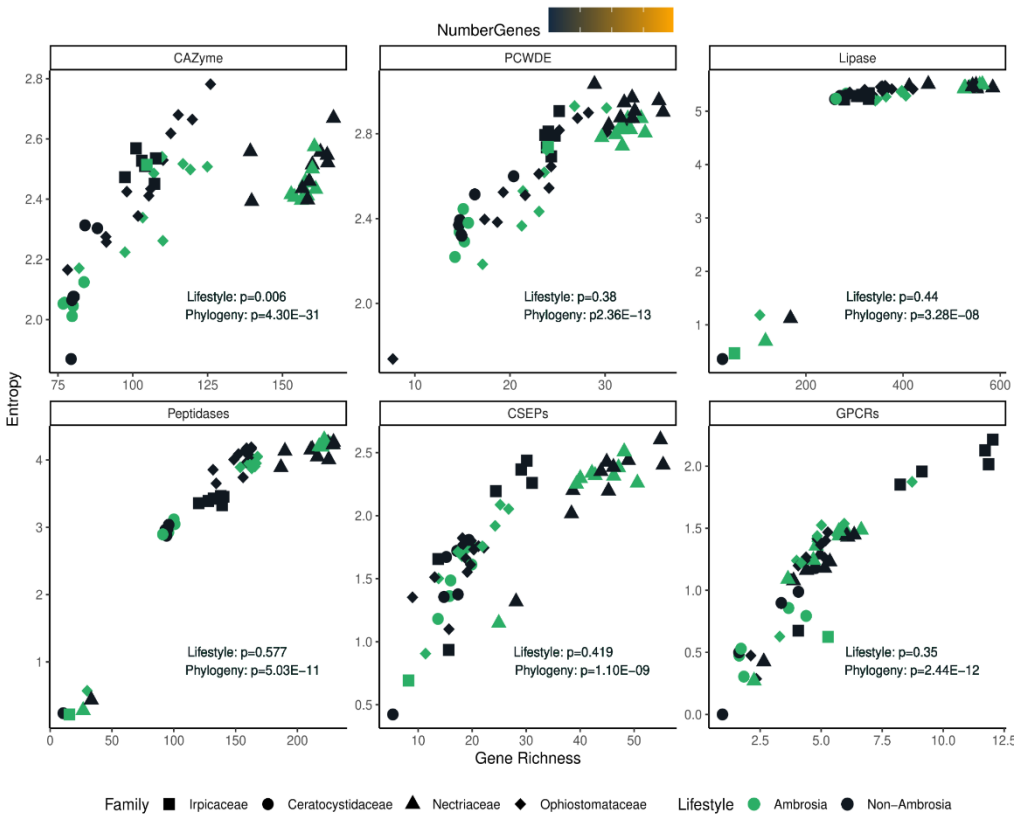


Figure 3. Gene repertoire dynamics in ambrosia fungi. (A) Gene copy number comparison between ambrosia and non-ambrosia fungi. Each point represents a gene family, with its position indicating mean copy numbers in ambrosia (y-axis) and non-ambrosia (x-axis) fungi. Color intensity reflects the number of genes at that ratio. Dashed line represents equal copy numbers. Inset values show total gains and losses per functional group in ambrosia fungi (gain:loss). (B) Functional diversity analysis. Each point represents a genome, with x-axis showing gene count and y-axis showing Shannon entropy diversity. Green dots indicate ambrosia fungi, black dots indicate non-ambrosia fungi. Point shapes denote fungal families: squares for Irpicaceae, circles for Ceratocystidaceae, triangles for Nectriaceae, and diamonds for Ophiostomataceae. P-values denote significance of diversity differences between ambrosia lifestyle (Lifestyle) or fungal family (Phylogeny) for each gene category.

Differential Evolution of CAZyme Families in Ambrosia Fungi

Our PIC analysis reveals family-specific enzyme evolution. Instead of observing a uniform trend across ambrosia lineages, we found distinct patterns of CAZyme family expansions and contractions within different fungal families, underscoring the necessity of considering phylogenetic relationships when interpreting these results (Table S5). In our overall analysis, we identified enzyme families such as AA4, CE4, and GH3 as having significant associations with the ambrosia lifestyle.

The AA4 family, which includes vanillyl-alcohol oxidases involved in lignin modification, showed a significant positive association with the ambrosia lifestyle in the PIC analysis (Estimate = 1.61, p = 0.011). Interestingly, this expansion is primarily driven by the ancestral non-ambrosial lineages within the Nectriaceae family, particularly in *Neocosmospora* species [*N. metavorans* (Al-Hatmi, S.A. Ahmed & de Hoog) Sand.-Den. & Crous, *N. liriodendri* Sand.-Den. & Crous, and *N. solani*). However, this expansion is subsequently reduced in the Nectriaceae ambrosia lineages. This pattern suggests that the expansion of AA4 may have been an important pre-adaptation in these lineages, potentially facilitating the later transition to the ambrosia lifestyle. The subsequent reduction in ambrosia lineages might indicate a shift in nutritional strategy or environmental adaptation as these fungi became more specialized for the ambrosia niche.

The CE4 family, which includes acetyl xylan esterases involved in hemicellulose modification, showed a significant expansion in the PIC analysis (Estimate = 0.81, p = 0.005). However, this expansion is particularly pronounced in the Ophiostomataceae ambrosia lineages compared to their non-ambrosia relatives. This family-specific expansion suggests that enhanced hemicellulose modification capabilities may be especially important for Ophiostomataceae ambrosia fungi, possibly reflecting differences in wood substrate preferences or gallery construction strategies among different ambrosia symbioses. Similar to the findings of De Fine Licht and Biedermann (2012), which highlighted the role of hemicellulolytic enzymes in fungus farming ambrosia beetles, our results indicate that Ophiostomataceae have adapted to optimize hemicellulose degradation in their symbiotic environment.

The GH3 family, which includes β-glucosidases crucial for cellulose degradation, showed a significant expansion in the PIC analysis (Estimate = 0.65, p = 0.046). Notably, this expansion is more prominent in both Ophiostomataceae and Ceratocystidaceae ambrosia

lineages. The expansion of GH3 in these two families suggests that enhanced cellulose degradation capabilities may be a shared adaptation in these groups of ambrosia fungi. This could indicate a convergent evolution towards more efficient cellulose processing, potentially allowing for better nutrient extraction from wood or manipulation of their gallery environment. The expansion of both CE4 and GH3 in Ophiostomataceae ambrosia fungi further suggests that this group may have evolved particularly enhanced capabilities for both hemicellulose and cellulose modification, supporting the conclusions of our enzyme activity analysis.

It is important to note that the signals from Irpicaceae ambrosia lineages are relatively weak in the overall PIC analysis, as only one genome from this group, generated in the present study, was included. This underscores the diversity of evolutionary strategies among ambrosia fungi and suggests that different fungal families may have adapted to the ambrosia lifestyle through distinct enzymatic modifications. The variability in microbial communities associated with ambrosia beetles at different life stages, providing diverse enzymatic functions and nutritional provisions, further supports the notion of varied evolutionary adaptations in this symbiosis (Ibarra-Juarez et al., 2020).

These results highlight the sophisticated and diverse nature of the ambrosia symbiosis across different fungal lineages, illustrating that the evolution of CAZyme repertoires in ambrosia fungi is a complex process that varies significantly among fungal families. Rather than a uniform loss or gain of wood-degrading functions, it appears that ambrosia fungi have evolved specialized enzymatic toolkits optimized for their unique ecological niches and phylogenetic backgrounds. The lineage-specific patterns of CAZyme evolution suggest that the transition to the ambrosia lifestyle may have occurred through different enzymatic adaptations in each fungal family, reflecting their diverse evolutionary histories and ecological contexts. The expansion patterns of AA4, CE4, and GH3 families provide evidence for specialized adaptations in lignin modification, hemicellulose deacetylation, and cellulose degradation, respectively. These findings indicate that the role of ambrosia fungi in wood modification may be more diverse and sophisticated than previously recognized, with different lineages adopting distinct strategies. Future research should focus on the biochemical characterization of these enzyme families within specific fungal lineages and their roles in different ambrosia beetle-fungus symbioses to fully understand their functional

significance in these fascinating ecological relationships.

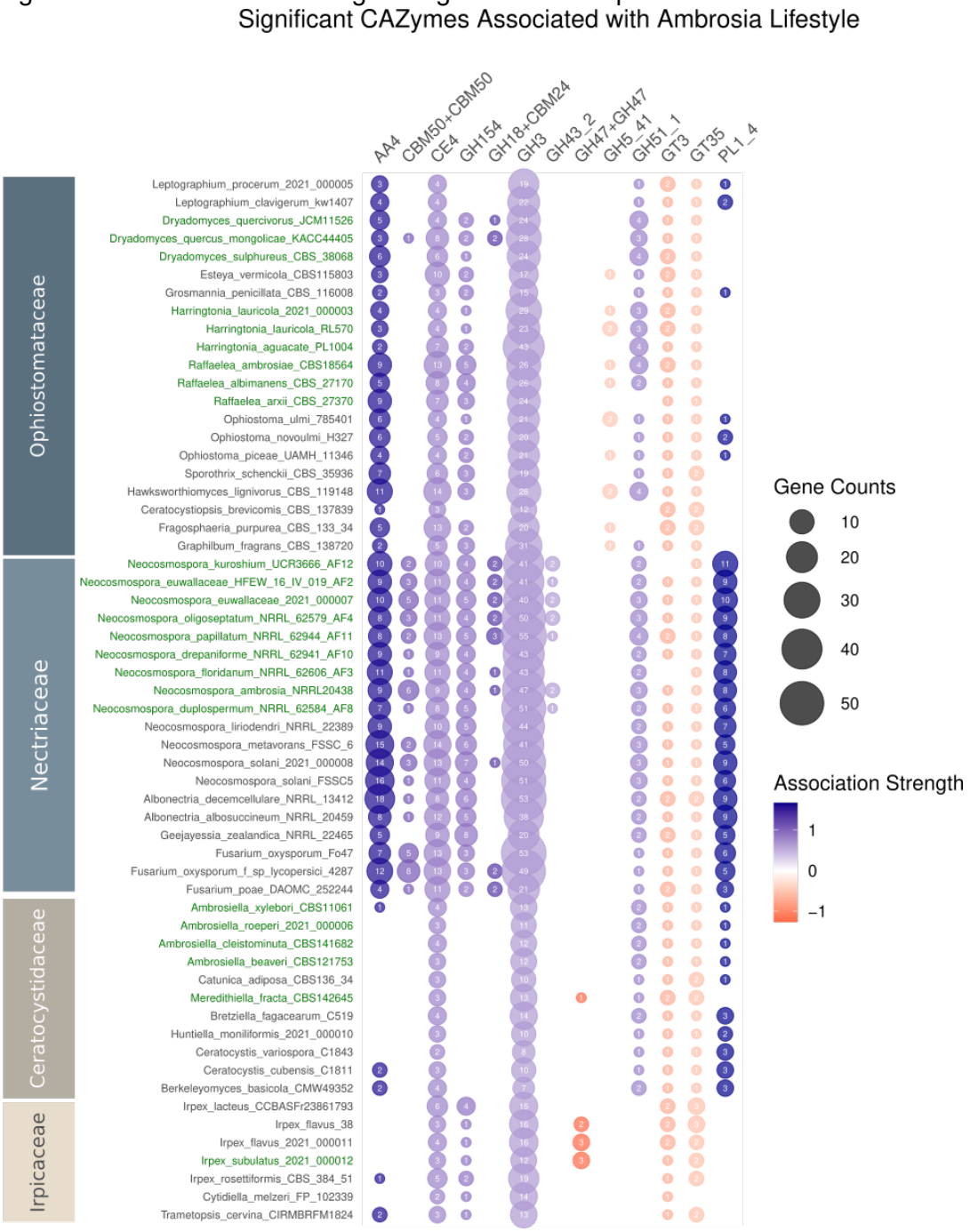


Figure 4. Bubble plot illustrating patterns of CAZyme enrichment and depletion across ambrosia fungi (green) and their non-ambrosia relatives (black). CAZyme families shown are significantly associated with ambrosia lifestyle ($p < 0.05$). Bubble size represents gene count per species. X-axis: CAZyme families, color-coded by association direction (blue: positive, red: negative), with color intensity indicating association strength. Y-axis: fungal species in the PIC test, ambrosia lineages are highlighted in green.

RIP Activity and Genomic Adaptation in Ambrosia Fungi

Repeat-Induced Point (RIP) mutations do not differ overall between ambrosia and non-ambrosia. Statistical analysis showed no difference in RIP-affected windows (Mann-Whitney U-test, $p = 0.9811$), RIP-affected genomic proportion ($p = 0.881$), or count of Large RIP Affected Regions (LRARs) ($p = 0.859$). This lack of statistical difference suggests that ambrosia lifestyle alone is not a primary determinant of RIP activity. While variations in RIP activity were observed across different fungal families, these differences cannot be attributed to the ambrosia lifestyle based on our statistical analysis. Most fungi in our dataset possessed the key methyltransferases DIM-2 and RID, as well as several cofactors involved in DNA methylation and heterochromatin formation, regardless of their ecology. Only *Dryadomyces quercus-mongolicae* and *D. sulphureus* lacked a detectable RID gene, while the free-living relative *Hawksworthiomyces lignivorus* lacked both RID and HP-1. The retention of these genes across most species, regardless of their ambrosia status or observed RIP activity, suggests that factors beyond symbiotic lifestyle influence the maintenance of these genomic elements. However, the presence of these genes alone does not guarantee RIP activity (Hane et al. 2015). Their maintenance in ambrosia fungi could indicate potential for cryptic sexual cycles (Mayers et al., 2017, 2020), additional gene functions beyond repeat silencing, or recent loss of RIP activity with insufficient time for gene loss.

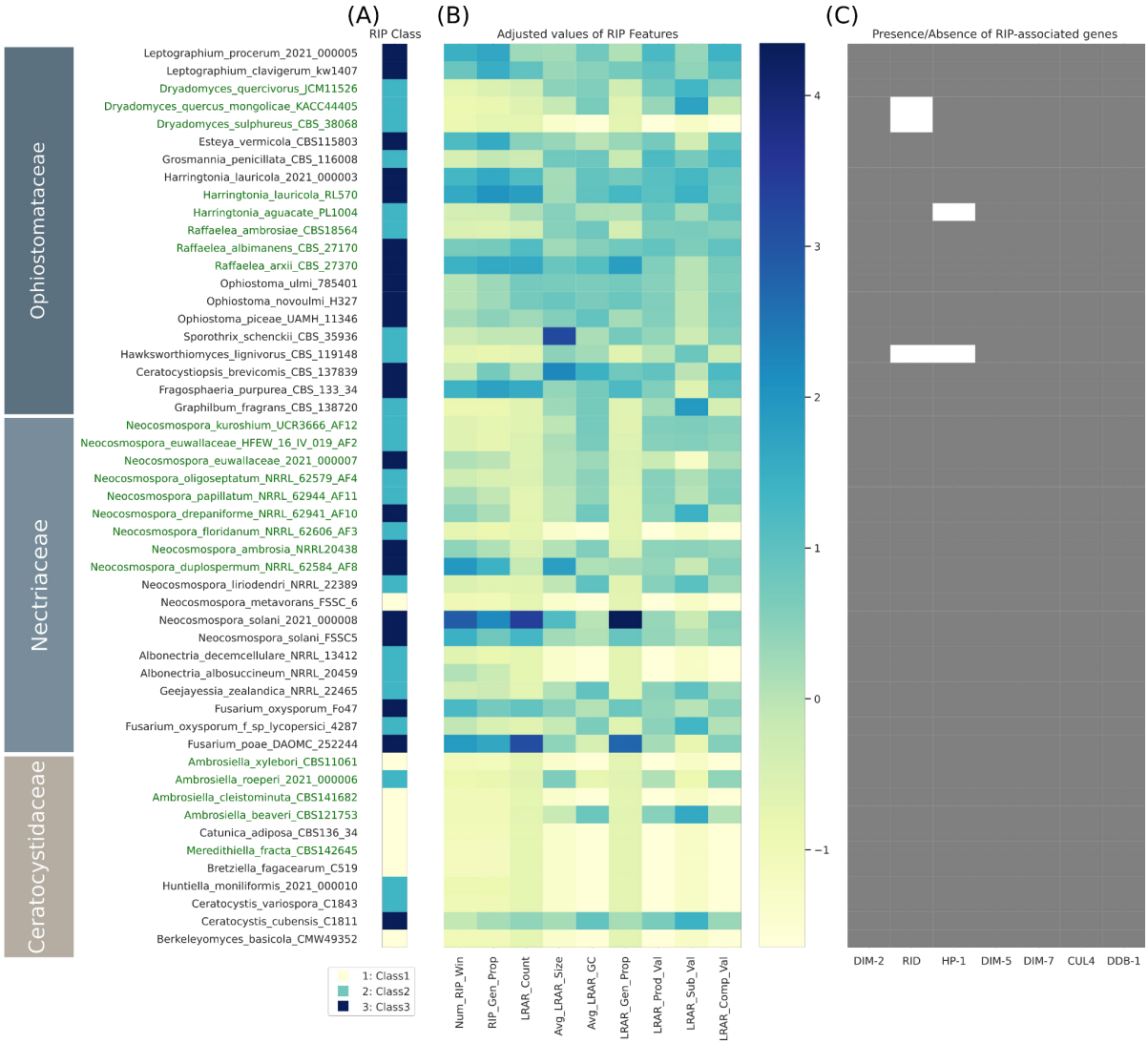


Figure 5. Genome-wide RIP analyses of ambrosia (green) and non-ambrosia (black) fungi. (A) RIP classes assigned based on the proportion of the genome affected by RIP, ranging from Class 1 (0–0.2%) to Class 3 (1–5%) (van Wyk et al., 2020). (B) Heatmap of standardized values for key RIP features: Number of RIP Affected Windows (Num_RIP_Win), RIP Affected Genomic Proportion (%) (RIP_Gen_Prop), Count of LRARs (LRAR_Count), Average Size of LRARs (bp) (Avg_LRAR_Size), Average GC Content of LRARs (%) (Avg_LRAR_GC), Genomic Proportion of LRARs (bp) (LRAR_Gen_Prop), Product Value for LRARs (LRAR_Prod_Val), Substrate Value for LRARs (LRAR_Sub_Val), and Composite Value for LRARs (LRAR_Comp_Val). (C) Presence (gray) or absence (white) of RIP-associated genes identified by blast against *Neurospora crassa* Shear & B.O. Dodge homologs. Irpicaceae species were excluded as RIP is primarily observed in Ascomycota.

Material and Methods

Fungal Strain Selection and Preparation

The fungal strains used in this study (Table S1) represent a spectrum of ecological associations within the ambrosia beetle system. Ambrosia fungi were defined as dominant and consistent mycangial isolates. We paired these with non-ambrosia counterparts selected based on phylogenetic relatedness and ecological relevance. These non-ambrosia fungi were isolated either from ambrosia beetle body surfaces or from associated wood substrates, allowing for comparative genomic analysis across different degrees of symbiotic association within similar ecological niches.

DNA Extraction and Quality Assessment

DNA was extracted from 10-day-old fungal cultures. Fungal tissues were scraped from PDA medium and placed into 1.5 ml microcentrifuge tubes containing 200 mg zirconia beads. The tubes were shaken using a Vortex-Genie 2 (Scientific Industries, Inc., NY, USA) at 3200 rpm for 4 minutes to disrupt the mycelium. DNA purification was carried out using a modified Bio-On-Magnetic-Beads (BOMB) protocol (Oberacker et al. 2019) with the ZiXpress 32 automated nucleic acid purification instrument (New Taipei City, Taiwan). DNA quality was assessed using an Invitrogen Qubit Fluorometer (ThermoFisher Scientific).

Genome Sequencing, Assembly, and Quality Assessment

Genomic DNA was sequenced using Oxford Nanopore Technology. Library preparation was performed using the Native Barcoding Kit 24 V14, following the manufacturer's protocol. We loaded 0.4 µg of DNA library onto an R10.4.1 flow cell and sequenced on a MinION device using MinKNOW software v23.07.5. Raw sequence outputs were basecalled using dorado v0.5.1 (github.com/nanoporetech/dorado) with the SUP model. Sequences were then processed using the nanoACT pipeline (github.com/Raingel/nanoACT). This processing included quality-filtering (q-score = 9, minimum length = 500, and maximum length = 1,000,000), demultiplexing using singlebar(), and trimming using trim_reads(). Trimmed reads were de novo assembled using Flye v2.9.3 with default settings and the "--nano-hq" option. Consensus sequences of the assemblies were polished using Medaka v1.8.1 https://github.com/nanoporetech/medaka) with Oxford nanopore reads. Transposable element (TE) coverage in the genomes was identified using RepeatModeler v2.0.5 and RepeatMasker v4.1.6. Genome completeness was assessed using BUSCO v5.6.1 with fungi-odb10 as a reference and default parameters. The resulting genome assemblies were deposited in NCBI under Bioproject accession PRJNA1084195 (Table S1).

Comparative genomic feature analyses

We performed genome annotation using BRAKER3 v3.0.5, which integrates RNA-Seq data from the Sequence Read Archive (SRA) with gene predictions from AUGUSTUS and GeneMark-ET. Gene functions were subsequently assigned using eggNOG-mapper v2.1.12 with the eggNOG orthology v5.0.2 database.

To predict the fungal secretome, we annotated our genomes using multiple databases. We identified signal peptides using SignalP 6.0 (Teufel et al., 2022), transmembrane domains using TMHMM via Galaxy (Krogh et al. 2001; Afgan et al. 2018), and subcellular localization using TargetP (Armenteros and Salvatore 2019). Proteins meeting any of these criteria were included in the predicted secretome. We further characterized the secretome for lipases and peptidases by aligning protein sequences to the Lipase Engineering Database (LED) (Fischer & Pleiss, 2003) and MEROPS peptidase database (Rawlings et al., 2018) using DIAMOND v2.1.8 (Buchfink et al., 2021). The top hit for each protein was selected based on bitscore. Carbohydrate-Active Enzymes (CAZymes) were annotated using dbCAN3 (Zheng et al., 2023) with HMMER, Diamond, and dbCAN_sub for CAZyme families annotation. Specifically, we only included annotated CAZymes genes that were concurrently annotated by both HMMER and dbCAN_sub. We categorized CAZymes into Plant Cell Wall-Degrading Enzymes (PCWDEs) following the classification scheme of Miyauchi et al. (2020). G-protein coupled receptors (GPCRs) were identified through a BLASTP search against the best hits in the GPCRDB database (Pándy-Szekeres et al. 2023),and their functional roles were cross-referenced with UniProt (UniProt Consortium 2023). To identify CSEPs, we used Effectorp 3.0. We retained both apoplastic and cytoplasmic effectors in our analysis, excluding only those identified as "non-effector" by the tool. All analyses were performed using default parameters unless otherwise specified.

We performed Multivariate Analysis of Variance (MANOVA) to examine the effects of ambrosia lifestyle and fungal family on diversity indices across various genomic tools. The analysis was conducted using the manova() in R. For each functional gene category, we carried out separate MANOVAs to assess the impact of ambrosia lifestyle and family on two diversity indices: gene richness and Shannon entropy. The Pillai's trace statistic was used to evaluate the significance of the effects.

Key genomic features were compared between ambrosia and non-ambrosia fungi: genome size, transposable element (TE) coverage, number of genes, scaffold N50, number of secreted proteins, and genomic GC content. To control for phylogenetic non-independence, we performed Phylogenetically Independent Contrasts (PIC) analysis using the 'ape' and 'phangorn' R packages. This method calculates contrasts in trait values between phylogenetically related species, accounting for shared evolutionary history. Linear models were then fitted to these contrasts, with genomic features as dependent variables and ambrosia lifestyle as the independent variable. These models quantify the association between each genomic feature and the ambrosia lifestyle while controlling for phylogeny, providing statistical estimates for each trait-lifestyle relationship.

Repeat-Induced Point Mutations Estimation

Repeat masked whole-genome sequences of ambrosia and non-ambrosia fungi were used to estimate Repeat-Induced Point (RIP) mutations in their genomes. RIP mutations were detected using The RIPper software (van Wyk et al., 2019) with stringent RIP parameters (RIP product > 1.1, RIP substrate ≤ 0.75, and RIP composite index > 7). The analysis employed a sliding-window approach with 1,000 bp windows and 500 bp step sizes. RIP-associated genes (DIM-2, RID, HP-1, DIM-5, DIM-7, CUL4, and DDB-1) were identified using BLASTp searches against these genes identified in *Neurospora crassa* (XP_959891.1, XP_957632.1, XP_011392925.1, XP_957479.2, XP_961308.2, XP_962347.1, and

XP_957743.3, respectively), with an E-value cutoff of 1e-5. Statistical analyses were performed using Python (version 3.10) with pandas, scipy, and seaborn libraries. The Mann-Whitney U test was used to compare RIP-related metrics between ambrosia and non-ambrosia fungi. Four key features were analyzed: number of RIP-affected windows, RIP-affected genomic proportion, and count of Large RIP Affected Regions (LRARs). Analyses were conducted both across all fungi and within individual fungal families.
Heatmaps were generated using seaborn and matplotlib libraries in Python. The heatmaps visualized RIP class, standardized RIP features, and gene presence/absence across fungal species. RIP classes were assigned based on the proportion of the genome affected by RIP, ranging from Class 1 (0-0.2%) to Class 6 (≥20%) according to (van Wyk et al., 2020). RIP features were standardized using sklearn's StandardScaler.

Phylogenetic Reconstruction

We used representative genomes of fungi across Basidiomycota and Ascomycota, including the most closely-related species to ambrosia fungi, along with two Mucoromycota representatives as outgroups, to reconstruct phylogenetic relationships. BUSCO v5.5.0_cv1 was run with the fungi_odb10 lineage database to generate single-copy orthologs from each genome. The shared single-copy orthologs were used as input for building the species tree. Sequences were aligned individually using MAFFT v7.490 with default parameters. The alignments were then trimmed using ClipKIT v2.3.0 with the "smartgap" option. We retained BUSCO gene alignments with ≥90% taxon occupancy for further analysis. Output alignments from ClipKIT were used as input to ModelTest-NG v0.1.7 with default settings to select the best evolutionary model for each gene. For the species tree reconstruction, we used a concatenation-based method. The set of retained 742 BUSCO gene alignments from MAFFT and ClipKIT were concatenated. The alignment was partitioned by gene, using the best evolutionary model as predicted with ModelTest-NG. We reconstructed the tree using IQ-TREE2 v2.0.7 with parameters "-bb 1000 -alrt 1000." The resulting tree was rooted to the Mucoromycota outgroups in FigTree. For the gene tree reconstruction, we analyzed each single-copy ortholog alignment separately. The same set of BUSCO gene alignments were processed using IQ-TREE2 with the evolutionary models predicted by ModelTest-NG, ensuring each gene tree was accurately reconstructed. All resulting gene trees were concatenated into a single file. ASTRAL v5.7.1 (Mirarab et al., 2014) was then employed to infer individual phylogenies from these BUSCO genes.

To test for significant discordance between gene trees and the species tree, we performed chi-square tests comparing the distribution of concordant and discordant gene tree topologies to the expectation under incomplete lineage sorting (ILS). For each branch in the species tree, we calculated the gene concordance factor (gCF) and site concordance factor (sCF) using IQ-TREE. We then conducted chi-square tests comparing the observed number of concordant vs discordant genes (for gCF) and sites (for sCF) to the expected 1/3 ratio under ILS. A custom R function was used to perform these tests for each branch, with p-values < 0.05 considered significant evidence of discordance beyond ILS expectations. Branches showing significant discordance in either gCF or sCF were identified as potentially resulting from processes other than ILS, such as introgression or incomplete taxon sampling.

Divergence Dating

To estimate the timing of ambrosial domestication events, we employed the RelTime method in MEGA X (Kumar et al., 2018), which calculates relative divergence times by leveraging molecular evolutionary rates across various branches of the phylogenetic tree (Dos Reis, 2022; Tamura et al., 2018). We input the concatenated alignments of single-copy BUSCO orthologs and the maximum likelihood (ML) topologies generated from our phylogenetic reconstruction. Our analysis incorporated three calibration nodes based on fossil records and the TimeTree database (Kumar et al., 2017): the divergence between Ascomycota and Basidiomycota (582 to 749 Million years ago [Ma]), the split between Sclerotiniaceae and Sordariomycetes (239.7 - 320 Ma), and a fossil record constraining the minimum age of Diaporthales (> 136 Ma) (Bronson et al., 2013). We modeled the molecular data using the Tamura-Nei model with gamma-distributed rates among sites (TN93+G), which was selected as the best fit based on Bayesian Information Criterion (BIC) scores.

Ancestral State Reconstruction

We performed ancestral state reconstruction (ASR) to infer the evolutionary history of fungus farming (ambrosia status) on the 61 taxa phylogeny inferred from our concatenated supermatrix dataset. The analysis was conducted using a maximum likelihood approach and stochastic character mapping. We used the ape (Paradis & Schliep, 2019) and phytools (Revell, 2012) packages in R for these analyses.

First, we used the ace() in the 'ape package to perform maximum likelihood ancestral state reconstruction. This method estimates the marginal ancestral state likelihoods for all nodes within the phylogeny, assuming that the probability of character state changes depends only on the current state and not on previous states. We applied the default equal rates (ER) model, which assumes equal transition rates between all character states. To account for uncertainty in the ancestral state estimates, we also conducted stochastic character mapping using the make.simmap function from the phytools package. This approach uses Markov chain Monte Carlo (MCMC) to sample character histories from their posterior probability distribution. We generated 1000 simulated character histories on the phylogeny using the ER model. The results of these simulations were summarized to obtain posterior probabilities of ancestral states at each node.

We visualized the results of both the maximum likelihood reconstruction and the stochastic character mapping on the phylogenetic tree. For the maximum likelihood results, we plotted pie charts at each internal node representing the probability of each ancestral state. For the stochastic character mapping results, we plotted a summary of the 1000 simulated character histories, with pie charts at nodes showing the proportion of simulations supporting each state.

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Legends

Table 1. Divergence dates of ambrosia lineages in the present study.

Ambrosia species	Family	Crown age (Ma)	CI_Lower (Ma)	CI_Upper (Ma)	Stem age (Ma)
Dryadomyces spp.	Ophiostomataceae	66.41	44.07	100.06	102.03
Harringtonia spp.	Ophiostomataceae	84.69	60.21	119.12	113.46
Raffaelea spp.	Ophiostomataceae	114.62	86.44	152.00	121.31
Neocosmospora spp.	Nectriaceae	9.37	5.80	15.14	11.82
Ambrosiella spp.	Ceratocystidaceae	14.31	9.90	20.70	60.08
Meredithiella spp.	Ceratocystidaceae	34.60	22.98	52.10	60.08
Irpex subulatus	Irpicaceae	1.94	1.58	54.23	9.24

Table 2. Phylogenetically Independent Contrasts (PIC) analysis results for genomic features in relation to ambrosia lifestyle. No significant associations were found after controlling for phylogeny

Trait	Estimate	StdError	tValue	pValue
Genome_size	1.13E+07	1.07E+07	1.053	0.296
TE_CoverageTotal	9.53E-01	4.61E+00	0.206	0.837
Num_Gene	1.70E+03	3.88E+03	0.436	0.664
GC_Genome	2.98E+00	3.15E+00	0.945	0.348
Secret_protein	5.70E+02	1.27E+03	0.450	0.654
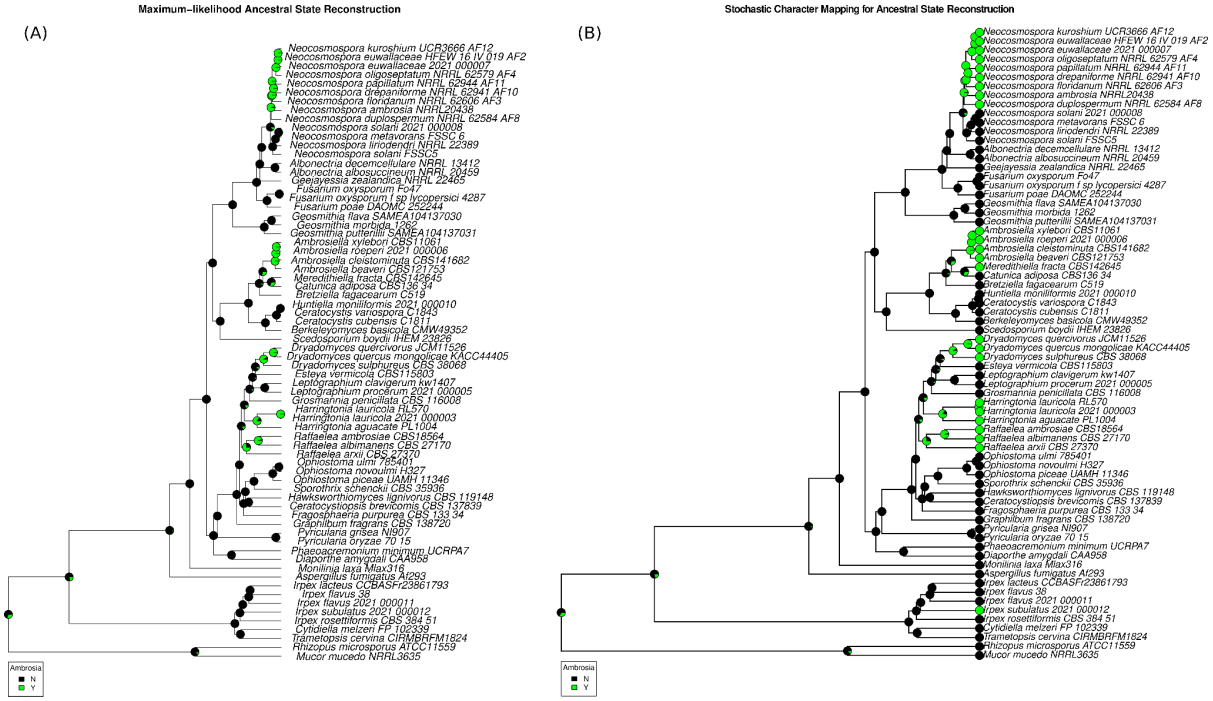
Figure S1. Ancestral State Reconstruction of Ambrosia Fungi