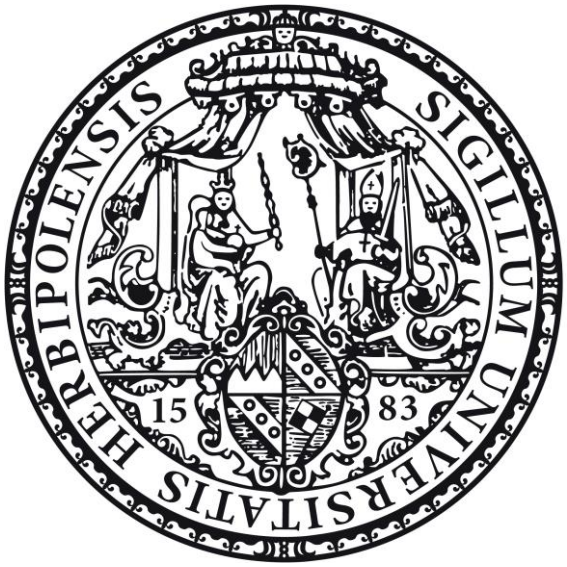

Ecology and evolution of symbiotic microbial communities in fungus farming ambrosia beetles

Ökologie und Evolution von symbiotischen mikrobiellen Gemeinschaften in Pilz-züchtenden Ambrosiakäfern

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Contents

General Introduction

Insights into wood-colonizing bark beetles (*sensu lato*)

Bark beetles (Curculionidae: Scolytinae) are a highly diverse group of insects, comprising around 6000 described species in 225 genera and in 25 tribes worldwide, where the majority of species occur in tropical and subtropical areas (Blackman, 1941; Raffa et al., 2015 and references therein). Bark beetles are colonizing healthy and/or dead or weakened woody tissues from the major groups of terrestrial plants all around the globe (Kirkendall et al., 2015). In fact, this wood-boring trait of weevils is quite old and originated multiple times at least around 90 – 120 million years ago (McKenna et al., 2009; Jordal et al., 2011; Haran et al., 2013; Gillett et al., 2014). For instance, the oldest fossil of a scolytine beetles is known from Labanese amber and is around 120 million years old (Kirejtshuk et al., 2009).

Bark beetles are generally characterized by excavating their galleries within almost all plant parts such as the outer bark (in case of *Dendroctonus brevicomis*), the phloem (in case of *Ips*) as well as the sapwood (in case of *Xyleborinus*), the roots (in case of *Pissodes* or *Hylastes*) and even the seeds (in case of *Hypothenemus*) (Blackman, 1941; Goheen et al., 1993; Damon, 2000; Six, 2003; Kirkendall et al., 2015; Raffa et al., 2015). Certain species are well known and utilized as important model systems for several scientific aspects like chemical ecology, symbiosis with specific microorganisms, population dynamics and disturbance ecology as well as studies on sexual selection and coevolution (see Blackman, 1941; Six, 2003; Raffa et al., 2015; Biedermann et al., 2019, Biedermann and Vega, 2020). Some species are even described as natural/ecological engineers, as their colonization not only causes death of trees, but includes several ecologically important effects such as gap and disturbance dynamics, canopy thinning, availability of dead wood, hydrology and nutrient cycling, causing increasing insect and plant diversity (Raffa et al., 2015; Müller et al., 2008; Stokland et al., 2012; Biedermann et al., 2019; Thorn et al., 2020).

However, certain bark beetles are competing with humans in terms of plants and their products. Besides lower impact within more natural mixed forests (conifers and broadleaf trees), this is especially the case in artificially planted monocultures (e.g. spruce) and plantations (e.g. avocado). Here, some species have the potential to cause high economic damages and are therefore considered as “pest” (see Kühnholz

et al., 2001; Hulcr and Dunn, 2011; Mendel et al., 2012; Raffa et al., 2015; Dohet et al., 2016; Biedermann et al., 2019), indicating the importance and the influence of certain bark beetles to humans. To minimize economic effects of bark beetle outbreaks by investigating possible management tools, some species are well studied. However, several aspects about their ecology as well as from the majority of remaining bark beetles remains open.

In fact, “bark beetle” is a rather generally applied term as it actually contains two life strategies: (**i**) the colonization of outer bark and phloem by bark beetles (*sensu lato*) and (**ii**) the colonization of sapwood (xylem) of woody plants by ambrosia beetles (*sensu stricto*), who originated from bark beetles (Biedermann and Vega, 2020). Indeed, phloem was the ancestral habitat for bark beetles in general, but after a nutritional mutualism with fungi (making life in xylem possible) originated, most lineages of solely fungus-feeding beetles (i.e. ambrosia beetles) moved from phloem to xylem (possibly because of high competitive pressure within phloem) (Kirkendall et al., 2015; Biedermann and Vega, 2020).

Based on their similar lifestyles, bark and ambrosia beetles are sharing several traits. They are mostly foraging in the dark, have to deal with several ubiquitous fungal antagonists, are highly adapted to their specific niche and are associated with various microorganisms like bacteria, yeasts, and filamentous fungi (see Kirkendall et al., 2015). Especially filamentous fungi are playing an important role in several bark and all ambrosia beetle systems as they can fulfill essential functions in these systems, e.g. serving as nutritional or tree-defenses-detoxifying mutualists and tree-defenses-stimulating fungi (e.g. Six, 2003; Kandasamy et al., 2019; Biedermann and Vega, 2020). Bark beetles are generally known to be associated with certain facultative mutualistic fungi, while ambrosia beetles have long been known for their species-specific, obligate nutritional mutualism with filamentous fungi (Kirkendall et al., 2015; Biedermann and Vega, 2020). Indeed, the role of the majority of associated fungi is still not entirely clear within several bark and ambrosia beetle systems. Some are known mutualists, others may have beneficial functions while the role of some frequently isolated species is still not clear. In ambrosia beetles, only about 5% of fungal associates were described so far (Hulcr and Stelinski, 2017), while the role of several other associated fungi, yeasts, and bacteria is even less studied (Haanstad and Norris, 1985). This indicates the importance of research on fungal associates of ambrosia beetles, but also on bark beetles, where even fewer species were

examined in detail in terms of their fungal associates (e.g. *Ips typographus*, *Dendroctonus* spp.).

Fungus-farming ambrosia beetles

The existence of fungus-farming ambrosia beetles is known since more than 180 years. In 1836, Josef Schmidberger, a monk who discovered ambrosia beetles for the very first time, noticed a gallery of a scolytine beetle within the sapwood in one of his apple trees, which tunnel system was covered with a whitish layer (Schmidberger, 1836). Based on his observations, he realized that this layer was nutritional for the beetles and - in his view - was provided by the trees to nurture the beetles. After the mythological food for the gods, he implemented the term “ambrosia” for this layer. Few years later, in 1844, it was the forester Hartig, who realized that this layer actually consists of fungal fruiting structures (Hartig, 1844). Since that time, these beetles are called “ambrosia beetles”.

Nowadays, the term “ambrosia beetle” is generally used for all xylem-boring and fungus-farming beetles in the curculionid subfamilies Scolytinae (bark beetles) and Platypodinae (pinhole borers) as well as the Lymexylidae (ship-timber beetles) (Neger, 1908). Here, the Platypodinae are classified as ambrosia beetles due to their fungus-farming and social behavior, but phylogenetically they are very likely more closely related to Dryophthorinae than to Scolytinae (but are still a highly derived group of weevils) (McKenna et al., 2009; Jordal et al., 2011; McKenna, 2011; Haran et al., 2013; Gillett et al., 2014; Kirkendall et al., 2015). Within the polyphyletic ambrosia beetles (encompassing more than 3,400 species worldwide) at least 13 evolutionary independent origins of fungus farming evolved (Hulcr and Stelinski, 2017; Biedermann and Vega, 2020).

Advanced fungus-farming involving active cooperative care of fungal cultures is not unique to ambrosia beetles. It evolved in three groups of insects: attine ants, macrotermites and wood-boring weevils in the subfamilies Scolytinae and Platypodinae (Farrell et al., 2001; Müller et al., 2002). But in contrast to attine ants and macrotermites that collect plant material, ambrosia beetles are colonizing the sapwood (xylem) of woody plants, which are typically recently dead or in an unhealthy state (Kirkendall et al., 2015). Indeed, colonizing woody tissue is a very challenging task. Sapwood is generally known to be chemically well defended in living, but also in weakened trees, and is an extremely nutrient-poor substrate, which is additionally biased towards certain elements, particularly carbon (C), hydrogen (H),

and oxygen (O) (Filipiak and Weiner, 2014; Pretzsch et al., 2014). To survive in such an extreme environment, ambrosia beetles are necessarily depending on species-specific mutualistic fungi, which they actively farm in often social family groups within their excavated galleries and which are serving as their essential and nutrient-rich source of food (Francke-Grosmann, 1967; Kirkendall et al,. 2015).

Such mutualistic fungi have repeatedly evolved from at least five ascomycete (Ophiostomataceae, Ceratocystidaceae, Nectriaceae, Bionectriaceae, Saccharomycetaceae) and two basidiomycete families (Peniophoraceae, Meruliaceae), and are known as descendants of mutualistic bark beetle fungi (Alamouti et al., 2009; Dreaden et al., 2014; Hulcr and Stelinski 2017; Vanderpool et al., 2018; Biedermann and Vega, 2020). Well known symbionts of ambrosia beetles are representatives of the two anamorph ascomycete genera Raffaelea and Ambrosiella, which interestingly originated from the plant pathogenic genera Ceratocystis and Ophiostoma, respectively (Cassar and Blackwell, 1996; Harrington et al., 2010). Moreover, several mutualistic species from both genera show exemplary adaptions in terms of their association with wood-boring beetles. These mostly polymorphic species change from a yeast-like growth within the beetles mycetangia (fungus-spore carrying structures, see below) to a filamentous growth within the beetle's galleries (see Batra, 1967). Inhere, ambrosia fungi line the tunnel walls with nutrient-rich asexual fruiting structures (conidia on conidiophores), which serve as the main food source for the beetles as well as their larvae, and are crucially needed to ensure their survival (Norris, 1979; Biedermann et al., 2013). Thus, ambrosia beetles are highly dependent on their mutualistic associates. The fungi in return highly depend on the presence of their beetle host as within the majority of the ambrosia beetle systems, antagonistic "weedy" fungi would overgrown them quickly if the beetles are not present (Kirkendall et al., 2015; Nuotclà et al., 2019).

Typically, the transportation of mutualistic fungi by the beetles is maintained over generations by vertical transmission of mutualistic symbionts. Here, the spores of fungi are carried in gland-rich pouches, the so-called mycetangia or mycangia, which are highly specialized and possibly selective structures (Francke-Grosmann, 1956; Batra, 1963; Six, 2003). The morphology and location of mycetangia differs between species (Hulcr and Stelinski 2017). However, detailed morphological studies on this structure using state-of-the-art-techniques (e.g. µCT see Li et al., 2018; Alba-Alejandre et al., 2019; Spahr et al., 2020) are rare. To date, the mechanisms that

maintain the transportation of fungi within mycetangia as well as underlying chemical pathways ensuring the survival of associated fungi mostly remains unknown.

Some physiological traits of bark and ambrosia beetle fungi and gaps of knowledge

Although the number of studies on bark and especially ambrosia beetles is constantly increasing in the past years (see Hulcr and Stelinski, 2017), several aspects still remain poorly investigated or even completely unknown for the majority of species. Indeed, most studies focused on various ecological effects of bark and ambrosia beetles in forest/plantations, but other branches of science (e.g. chemical ecology, coevolution, as well as studies on associated microorganisms) are becoming more and more important. Still, only few studies focused on the physiology of fungal symbionts of bark and ambrosia beetles, although this knowledge is crucial to understand how the beetle-fungus symbioses is maintained, and thus to answer several open ecological and evolutionary questions. Gaining novel insights into this poorly understood symbiotic world between these beetles and their associated filamentous microbes would not only help to understand why and how they evolved certain traits, but also assist in the development of possible management tools by targeting particularly the symbiotic fungi.

Wood-degradation by ambrosia beetle fungi

To date, research dealing with metabolic characteristics of ambrosia beetle fungi is rare. Here, only few studies examined the availability of certain ambrosia beetle fungi to metabolize different carbon sources such as xylose, dextrose or cellobiose (Batra, 1967; Norris and Baker, 1968). A recent study showed that the few tested ambrosia beetle fungi were able to utilize several simple sugars (from monosaccharides to polysaccharides) and polymers as well as certain amino acids and amides as a carbon source, while some substances such as L-fucose, D-psicose, and 2-3-butanediol were only hardly metabolized (Huang et al., 2019). Indeed, De Fine Licht and Biedermann (2012) conducted the first extensive enzymatic investigation of an ambrosia beetle system by examining an entire gallery of *Xyleborinus saxesenii*. They showed that microbial associates occurring within the gallery are able to depolymerize certain hemicelluloses such as xylan, glucomannan, and callose. Currently, it is believed that the majority of ambrosia beetle fungi does degrade plant polymers such as cellulose or lignin (Kim et al., 2011; Veselská et al., 2019), but detailed studies examining the enzymatic capabilities of ambrosia beetle fungi to

decompose the main woody polysaccharides using a more natural wood-based medium are absent.

Competition with antagonistic fungi

Ambrosia beetle fungi somehow must accomplish several tasks to gain nutrients out of colonized substrate, which is only possible if they are among the most dominate beneficial fungi within the beetle's gallery. It is known that mutualistic fungi highly depend on the presence of the beetles as otherwise they would be overgrown by competitive fungi (Kirkendall et al., 2015; Nuotclà et al., 2019). Apparently, also other possible mechanisms, which may maintain the symbiosis between beetles and fungi, have evolved. A recent study showed that certain mutualistic ambrosia beetle fungi are utilizing the antimicrobial compound ethanol for an environmental screening within the galleries they are cultivated (Ranger et al., 2018). Here, the authors could show that ethanol, which is (together with other alcohols) emitted by ambrosia beetle fungi (Kuhns et al., 2014; Kandasamy et al., 2016) as well as by stressed but also healthy colonized host trees (e.g. Kimmerer and Kozlowski, 1982; Kimmerer and Stringer, 1988; Kimmerer et al., 1991; Kelsey et al., 2014), strengthens the ability of mutualistic ambrosia beetle fungi to compete with antagonistic "weedy" fungi, while such antagonists were highly inhibited by even low concentrations of ethanol (Ranger et al., 2018). The tested ambrosia beetle fungi were apparently even able to utilize ethanol as a carbon source or at least as a growth inducer, as they produced more biomass compared to fungi cultured without ethanol. Ethanol is generally known to be of high importance to the beetles as it has a crucial role for the attraction of ambrosia beetles to their host trees, and thus supports them to find deficient trees (Graham, 1968; Kühnholz et al., 2001; Ranger et al., 2015). Besides the few tested fungi in Ranger et al. (2018), it remains unclear, if the observed ethanol tolerance and its possible metabolization also occurs within other related and non-related mutualistic ambrosia beetle fungi. Moreover, it would be interesting to know if this pattern also occurs within fungi associated with phloem-colonizing bark beetles (evolutionary ancestors of ambrosia beetles), as they still prefer similarly deficient host trees. To date, it is unknown if the ethanol naturally occurring within the phloem of host trees (MacDonald and Kimmerer, 1991) also has beneficial effects towards bark beetle fungi.

Surviving within an extremely nutrient-poor environment

In contrast to bark beetles, which are colonizing the nutrient-rich phloem (or bark), ambrosia beetles are drilling their tunnel systems within the xylem of trees, which is an extremely poor source of nutritional element (Pretzsch et al., 2014; Kirkendall et al., 2015; Ulbricht et al., 2016). Bark beetles and their associated fungi might benefit from this trait, while it indicates the challenging situation ambrosia beetle fungi somehow must overwhelm to ensure that they can produce enough nutrient-rich biomass/fruiting structures to nurture the beetles and larvae. All insects feeding in woody substrates highly depend on the availability of certain elements such as nitrogen (N), sulfur (S), and phosphorous (P), which occur in wood only in very low amounts, but are essential for several biological functions (Fengel and Wegener, 1989; McDowell, 2003; Filipiak et al., 2016). Indeed, around 99% of this tissue typically consists of C, H and O, while the remaining 1% is mostly made out of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and sodium (Na) (Sterner and Elser, 2002; Fengel and Wegener, 1989; Filipiak and Weiner, 2014). In such an environment, the association with beneficial microbes supports several insects, as they would be unable to gather enough elements without them (Buchner, 2013). Therefore, insects colonizing woody tissues often utilize microbes to enrich their diet (e.g. Täyasu et al., 1994; Ayayee et al., 2014; Filipiak and Weiner, 2017). Fungi, which have the capability to grow far into the woody tissue may acquire the rare elements and could, in case of ambrosia beetle fungi, provide these to beetles and larvae. Currently, it is unclear how far ambrosia beetle fungi are growing into the wood. Some species are believed to forage only few millimeters inside the xylem, while others may have the capability to grow up to 20 centimeter inside the stump (Francke-Grosmann, 1966), but no experiments have proved their abilities yet. As ambrosia beetles typically only feed inside their galleries, all required elements must come from xylem surrounding the colonized gallery (N may be an exception, see Peklo and Satava, 1949). However, it is currently unclear how ambrosia beetle fungi are providing nutrients to their beetle hosts. A recent study revealed that mutualistic fungi associated with the bark beetle *Dendroctonus brevicomis* are concentrating and translocating elements such as N and P from the xylem to phloem and to the outer bark, where the beetles and larvae feed (Six and Elser, 2019). Here, the authors applied ecological stoichiometry for the very first time within a bark beetle system. Briefly, this branch of science briefly describes the movement and balance/imbalance

of elements within natural systems as well as the influence of ecological processes (Sterner and Elser, 2002). As this method allows to investigate availability and limitations of elements, it is a useful tool to get an overview of tropic relations in general, and thus can be used to examine certain nutritional mechanisms in insect-microbe mutualisms.

Volatile organic compounds (VOC) emitted by beetle-associated fungi

Filamentous fungi are playing a crucial role within bark and ambrosia beetle systems and are thus protected by their hosts. Especially ambrosia beetles are well known for their active care and farming behavior towards their mutualistic fungi (Francke-Grosmann, 1966; Biedermann et al., 2013; Biedermann and Vega, 2020). As both bark and ambrosia beetles are mostly foraging in the dark (in phloem or xylem) (Kirkendall et al., 2015), certain mechanisms must have evolved to ensure that beetles can distinguish between beneficial mutualistic fungi and ubiquitous fungal competitors, which have the potential to overgrow beneficial fungi within the galleries (see Nuotclà et al., 2019) and even infest and kill the beetles (in case of entomo-pathogens, e.g. *Beauveria bassiana*). Indeed, some ambrosia beetles were already described to be attracted by their fungal associates through emitted VOCs, but individual compounds were not identified (Hulcr et al., 2011). To date, only very few ambrosia beetle fungi were examined in terms of their VOCs (Francke and Bruemmer, 1978a,b; Kuhns et al., 2014; Egonyu and Torto, 2018; Cooperband et al., 2017), while these compounds as well as their possible attractive or repellent effects remain unstudied for the majority of known species. A recent study intensively examined attractive and repellent effects of fungi associated with the spruce-infesting bark beetle *Ips typographus* (Kandasamy et al., 2019). Here, the authors showed that beetles are significantly attracted by some species, while others have repellent effects. Additionally, they identified several VOCs emitted from tested fungi including aliphatic and aromatic esters and alcohols as well as certain mono- and sesquiterpenes. Testing artificial blends of volatile organic compounds (based on emitted volatiles from examined fungi), they proved that these VOCs are responsible for attractive/repellent effects towards the beetles. Similar to ambrosia beetle systems, fungal volatile organic compounds and their role within bark beetle systems are only studied for few species (for bark beetles see summary in Kandasamy et al., 2016), and several systems remains poorly investigated. Indeed, fungal VOCs may have the potential to act as key players in maintaining the symbiosis within bark and

ambrosia beetles with their mutualistic fungi and therefore makes further studies necessary.

Research aims

Within my PhD project “Ecology and evolution of symbiotic microbial communities in fungus-farming ambrosia beetles” I generally aim to tackle long-standing questions focusing on physiological mechanisms of beetle-associated fungi that may maintain the symbiosis between the beetles and their fungal mutualists, which are currently poorly understood. Therefore, I investigated a broad range of ambrosia beetle fungi from convergently evolved phylogenetic lineages. To understand, compare, and highlight evolutionary mechanisms within fungus-associated scolytinae, I included several free-living, non-mutualistic fungi as well as fungal bark beetle associates (which are ancestral to ambrosia beetle fungi) from the same phylogenetic lineages within my individual projects as well as within phylogenetic analyses.

Physiology and maintenance of the *Trypodendron* – *Phialophoropsis* symbiosis

(**i**) Ambrosia beetle fungi are currently believed to be rather poor wood decomposers, but experiments investigating the wood-degrading capabilities of individual ambrosia beetle associated fungi are missing. Therefore, I aimed to examine the wood-decomposing potential of fungal mutualists associated with ambrosia beetles grown on wood meal by analyzing secreted glycosyl hydrolases using supernatant-based enzymatic assays for the main woody polysaccharides. Moreover, as the association between *Trypodendron* ambrosia beetles with mutualistic fungi in the genus *Phialophoropsis* had not been safely established, I aimed to confirm the association of *Trypodendron* with *Phialophoropsis* in general and additionally to examine possible species-specific differences in terms of the associated mutualistic fungus.

(**ii**) These mutualistic fungi are transported in highly specialized organs (mycetangia) by the beetles, but only few studies provided a detailed morphological description of such spore-carrying structures using state-of-the-art-techniques. To improve our understanding of fungal transportation in mycetangia, I aimed to investigate the mycetangium of a widespread but rare European ambrosia beetle using micro-Computed Tomography (µCT).

Physiological capabilities of ambrosia fungi

(**iii**) A recent study determined beneficial effects of ethanol towards certain ambrosia beetles and especially their fungi (see Ranger et al., 2018). As the authors included only few species, I aimed to expand this study to a broader range of former unstudied species of bark and ambrosia beetle fungi as well as to few free-living non-mutualists using a culture dependent method.

(**iv**) Ambrosia beetles are typically only feeding within their galleries, which they drill into the extremely nutrient-poor sapwood of trees. Several crucially needed elements are only available in very low proportions but still ambrosia beetle fungi are an excellent source of nutrition for their beetle host. To tackle this question, I aimed to investigate the possible enrichment of several elements within the galleries of a few independently involved ambrosia beetle systems, what may be caused through the translocation of elements from the surrounding xylem by mutualistic filamentous fungi (possibly similar as described by Six and Elser, 2019). Therefore, I applied scanning electron microscopy in combination with energy-dispersive X-ray spectroscopy (SEM-EDX) to beetle galleries for the very first time within an ambrosia beetle system. Additionally, I utilized inductively coupled plasma optical emission spectroscopy (ICP-OES) to quantify involved elements within the beetle's galleries.

Volatile organic compounds of beetle associated fungi

(**v**) Both bark and ambrosia beetles are foraging almost completely in the dark. Moreover, a recent study showed that volatile organic compounds emitted from associated fungi are key players in maintaining the beetle-fungus symbiosis of *Ips typographus*, as they allow to distinguish between beneficial and competitive fungi (see Kandasamy et al., 2019). However, detailed studies dealing with the identification of fungal volatile organic compounds in bark and especially ambrosia beetle systems are missing, though. Therefore, I aimed to examine and compare the presence of such compounds over time within a broad range of independently evolved bark and ambrosia beetle fungi including free-living non-mutualists in the same phylogenetic lineages using a culture-dependent method. For semi-quantitative analysis, I applied gas chromatography coupled to a quadrupole mass spectrometer with a thermal desorption unit and a Cryo-Trap (GC-MS-TDU). Moreover, I aimed to profile the amino acid content of these fungi (crucially needed to produce volatiles) over time with a culture-dependent method, utilizing liquid chromatography – mass

spectrometry (LC-MS/MS), what may additionally provide novel insights into the nutritional quality of both bark and ambrosia beetle fungi.

Chapter 1

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Molecular identification and enzymatic profiling of *Trypodendron* (Curculionidae: Xyloterini) ambrosia beetle-associated fungi of the genus *Phialophoropsis* (Microascales: Ceratocystidaceae)

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ABSTRACT

Ambrosia fungi are a polyphyletic group from currently seven ascomycete and basidiomycete lineages that independently evolved an obligate farming mutualism with wood-boring weevils. One long known, but understudied, association is the mutualism between the scolytine beetle genus *Trypodendron* (Curculionidae: Xyloterini) and the Microascales fungal genus *Phialophoropsis* (Ascomycota: Ceratocystidaceae) for which a species-specific association has not been safely established yet. Moreover, the fungal wood degrading capabilities are completely unknown.

Here, the ambrosia fungi of three Xyloterini species, *Trypodendron domesticum, Trypodendron lineatum* and *Trypodendron signatum,* were isolated and identified using culture-dependent methods. *T. lineatum* was confirmed to be exclusively associated with *Phialophoropsis ferruginea,* whereas *T. domesticum* and *T. signatum* are associated with a closely related but putatively novel *Phialophoropsis* species. Investigations of their wood decomposing potential revealed that both fungi mainly depolymerize xylan but are weak mannan decomposers. In addition, robust cellulolytic activity was observed, indicating cellulose as another main carbon source.

1. Introduction

Fungus-farming evolved in three groups of insects: attine ants, macrotermites and wood-boring weevils in the subfamilies Scolytinae and Platypodinae (Farrell et al., 2001; Muller et al., 2005). Farming weevils, the so-called ambrosia beetles, currently encompass more than 3400 species in at least eleven lineages that independently evolved a nutritional mutualism with fungi (Hulcr et al., 2007). All ambrosia beetles are dependent on the presence of woody plants, which are typically recently dead or in an unhealthy state, in which they drill their tunnel systems ("galleries"). Therein, they actively farm one or several fungal mutualists, which serve as their essential source of food (Francke-Grosmann, 1967; Kirkendall et al., 2015).

Analogous to the polyphyletic ambrosia beetles, fungal mutualists – the so-called ambrosia fungi – have repeatedly evolved from at least five ascomycete (Ophiostomataceae, Ceratocystidaceae, Nectriaceae, Bionectriaceae, Saccharomycetaceae) and two basidiomycete families (Peniophoraceae, Meruliaceae) (Hulcr and Stelinski, 2017). While an earlier hypothesis suspected tight coevolution and species-specificity between beetles and fungi (Francke-Grosmann, 1967; Muller et al., 2005), current data suggests that fungal lineages are switched between beetle lineages (e.g. Vanderpool et al., 2017) and that the tightness of the association depends on the beetle taxon (Kostovcik et al., 2014; Mayers et al., 2015). The best studied Xyleborini ambrosia beetle lineage, for example, encompasses apparently tightly evolved associations in the genera *Anisandrus*, *Xylosandrus* and *Xyleborinus*, but also

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more flexible beetle-fungus associations in the genus *Xyleborus*. However, the causes for these potential switches of the symbionts are unknown. Possible reasons are unspecific vertical transmission of fungi between natal and newly founded nests and/or horizontal fungal exchange between neighboring nests when different beetle species colonize the same host tree (Bateman et al., 2015).

Typically, partner fidelity between beetles and fungi is maintained over generations by vertical transmission of fungal symbionts in mycetangia, which are gland-rich pouches that are specialized for fungal spore transmission and thought to be more or less selective (Francke Grosmann, 1956; Batra, 1963; Six, 2003). The morphology and location differs between species, and mycetangia can be located internally or externally (Hulcr and Stelinski, 2017).

Within the galleries, ambrosia fungi line the tunnel walls with asexual fruiting structures (conidia on conidiophores), which serve as the main food source for the beetles and their larvae (Norris, 1979; Biedermann et al., 2013). To date, only about 5% of the fungal symbionts of known ambrosia beetle species have been described (Hulcr and Stelinski, 2017). Even less studied is the identity and role of other mycelial fungi, yeasts and bacteria that are frequently found to co-inhabit the ambrosia beetle galleries (Haanstad and Norris, 1985).

Hitherto, little is known about the metabolic characteristics of ambrosia fungi in general. A few studies investigated the utilization of different carbon sources by some ambrosia fungi. For instance, the ambrosia fungi *Raffaelea brunnea* and *Raffaelea tritirachium* are known to assimilate various carbon compounds like xylose or cellobiose (Batra, 1967). Using a minimal nutrient assay, the fungal symbiont of *Xyleborus ferrugineus* was found to metabolize dextrose but not cellulose (Norris and Baker, 1968). The first extensive enzymatic profiling of an ambrosia association investigated the whole gallery of *Xyleborinus saxesenii* and showed the ability to depolymerize hemicellulose, such as xylan, glucomannan and callose (De Fine Licht and Biedermann, 2012). Besides these findings, we are unaware of any study examining the enzymatic activity of individual ambrosia fungi on a wood-based medium, elucidating the fungal ability to utilize the wood polysaccharides.

The main purpose of this study was to examine the specificity and fungal physiology of the *Phialophoropsis-Trypodendron* mutualism. In particular, we focused on the fungal mutualists of *Trypodendron lineatum*, *Trypodendron domesticum* and *Trypodendron signatum*, which were not all safely identified, and for which enzymatic profiles are unknown. Best studied is *T. lineatum*, for which *Phialophoropsis ferruginea*, comb. nov. was identified as the fungal symbiont a long time ago (Mathiesen-Käärik, 1953; Francke-Grosmann, 1956; Mayers et al., 2015). More recently, another *Phialophoropsis* sp. was isolated from *T. domesticum* (Mayers et al., 2015) and a fungus with similar conidiophore morphology to *P. ferruginea* from *T. signatum* (Nakashima et al., 1992). However, most of the fungal species were isolated out of only one or just a few beetles (or numbers of examined beetles were not reported). Moreover, as all three beetles occur in the same forest habitats, it is possible that they switch their *Phialophoropsis* associates.

To test for specificity and potentially associate each beetle with its respective symbiotic fungus, sufficiently large numbers of ambrosia fungi were isolated from *T. lineatum*, *T. domesticum* and *T. signatum* collected in the same habitats. All fungal isolates were sequenced for their ITS and in parts for their TEF1α region and were then used for phylogenetic analyses. Furthermore, the major enzymatic activities necessary to decompose wood as the natural host material were investigated for individual fungi. Finally, an examination of the fungal morphology of two *Phialophoropsis* species illustrated an amazing variability that had been noticed before (Leach et al., 1940) but had not been further described nor investigated.

2. Materials and methods

2.1. Beetle collections

Beetles were caught in March of 2017 with pheromone-baited traps ("Lineatin Kombi" by Witasek, Germany; Suppl. Fig. 1 A & B) during 5 d sampling at the Bavarian Forest National Park (Neuschönau, Böhmerweg, between 850 and 966 m NHN, Germany) and 11 d sampling in the "Plantage" in Freising (between 497 and 513 m NHN, Germany). Subsequently, the beetles were individually stored in 1.5 ml Eppendorf tubes filled with a piece of damp tissue and transported to the lab for morphological identification (Grüne, 1979; Bussler and Schmidt, 2008). Individual beetles were kept in the cooling room (3–6 °C) and fungi were isolated immediately after beetle identification.

Active *Trypodendron* galleries in beech and fir (*Fagus sylvatica, Picea abies*) were identified and excised with a chainsaw in June 2017 in the Bavarian Forest National Park (Suppl. Fig. 1C & D) at the same locations where the pheromone-baited traps were placed. The wood pieces containing the beetle's galleries were subsequently cooled and transported to the lab.

2.2. Artificial rearing of beetles

Some of the caught beetles were used for lab-rearing on a modified sawdust-agar substrate (Biedermann et al., 2009; Suppl. Fig. 1 J). Briefly, the following ingredients were used for 12–14 50 ml conical Falcon tubes: 100 g sawdust (spruce, beech or oak), 0.63 g Wesson salt mixture (W1374 Sigma-Aldrich), 2.5 g yeast-extract, 15 g agar, 280 ml water, 5 ml wheat-germ oil and 4 ml of ethanol (absolute). In contrast to the reported medium, addition of any simple sugars to stimulate fungal growth was omitted. After autoclaving of the half-liquid media and some minutes of cooling, the ethanol was added, mixed again and finally distributed into sterile tubes. The media inside the tubes was compacted with a sterile spatula and dried for 2 d. The surface of collected *Trypodendron* females and males were briefly sterilized with 70% ethanol to reduce the potentially vectored contaminants (e.g. molds), followed by a short rinse in sterile, deionized water. Finally, the females were individually placed into the tubes, which subsequently were loosely closed, horizontally stored and incubated in ambient conditions such that only the first 5 cm would be exposed to light. One male per tube was added after 0–6 d, after they were sterilized as mentioned above. The purpose of this rearing was not to breed beetles, but to isolate fungi from the galleries. The ambrosia fungus was isolated out of the galleries after the fungus colonized the entire medium inside the tube (after 10–20 d of incubation) by scraping off some of the fungal biomass with a sterile wood stick, after the tubes were cut in two pieces with a sterilized knife.

2.3. Fungal isolations

Fungal isolates were obtained from the excised mycetangium or the whole prothorax (including the mycetangium) and the gut of individual beetles by plating out these squashed body parts in PBS buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; 0.1% Tween-20, pH 7.4) in different dilutions (undiluted, 1:10, 1:100, 1:1000) and incubating at room temperature. Two types of solid media (YEMA 1: 0.3% yeast-extract, 1% malt-extract, 1.75% agar; YEMA 2: 0.5% peptone, 0.3% yeast-extract, 0.3% malt-extract, 1% D-glucose, 2% agar) were used and supplemented with 50 μg/ml streptomycin (Sigma-Aldrich, Germany) after autoclaving.

Fungal isolations out of the field galleries (Suppl. Fig. 1 E–I) and beetle rearing media (Suppl. Fig. 1 J) were obtained by scraping off some of the fungal biomass with a sterile wood-stick and

transferring it to YEMA 1 or 2.

These isolates were first separated into possible ambrosia fungi and other filamentous fungi by visual inspection, which was possible due to their rather distinctive morphology (Fig. 1): In the initial state, the majority of all ambrosia isolates had a red center followed by a whitish outgrowth (Suppl. Fig. 2, 7d, A-D). Furthermore, fungal mycelia were limited to the surface of the plates and showed a rather compact morphology (Fig. 1 B, D, F).

2.4. DNA extraction and ITS/TEF1α barcoding

All isolates with *Phialophoropsis* morphology were identified by barcoding. DNA extraction followed the Epicentre MasterPure™ Yeast DNA Purification Kit or the Jena Bioscience - Animal and Fungi DNA Preparation Kit. Before DNA extraction, the fungal samples were homogenized by grinding in liquid nitrogen. For PCR, the master-mix used was identical for ITS and TEF1α amplifications (Master-mix for 50 µl: 10 µl Phusion buffer HF 5x, 1 µl dNTP-mix (10 mM each), 2.5 µl primer 1 (10 µM), 2.5 µl primer 2 (10 µM), 0.5 µl Phusion polymerase, 1 µl template (usually 1:100 diluted)) (New England Biolabs, Germany).

The internal transcribed spacer (ITS) region of the ribosomal DNA was used for the identification of each fungal species. For DNA amplification, a Ceratocystidaceae-specific primer (Cerato1F: GCGGAGGGATCATTACTGAG) was used (Mayers et al., 2015) in combination with a general fungal primer (ITS4-R: TCCTCCGCTTATTGATATGC) (White et al., 1990). But also the common primer combination for the ITS region, consisting of ITS1-F (TCCGTAGGTGAACCTGCGG) and ITS4-R were used (White et al., 1990). The following PCR protocol was applied: 98 °C for 30 s, followed by 35 cycles of 98 °C 10 s, 54.5 °C 30 s and 72 °C for 20 s, ending with 72 °C for 10 min and cooled down to 15 °C. The resulting sequence lengths were about 580 bp.

The translation elongation factor 1-alpha (TEF1α) was amplified using primers EFCF1a (F: AGTGCGGTGGTATCGACAAGCG) and EFCF6 (R: CATGTCACGGACGGCGAAAC) (Harrington, 2009) with a PCR protocol adapted to Phusion polymerase (on the basis of Mayers et al., 2015 and Oliveira et al., 2015): 98 °C 30 s, followed by 35 cycles of 98 °C 10 s, 62 °C 30 s and 72 °C for 30 s, followed by 72 °C for 5 min and ending with a cooling to 15 °C. The TEF1α-sequences finally contained approximately 900–1000 bp. After gel electrophoresis DNA-purification was performed using the Wizard® SV Gel and PCR Clean-Up system (Promega, Germany). The sequencing was accomplished by Eurofins (Eurofins Genomics, Germany). The identification of the fungal species was done using BLASTn at NCBI (Altschul et al., 1990).

2.5. Phylogenetic analyses

For the phylogenetic analyses, sequences of the TEF1α and ITS were used for the construction of phylogenetic trees. Six GenBank entries for the ITS dataset (Fig. 3) and three GenBank entries for the TEF1α (Fig. 4) dataset were used for comparison (Table 1).

The sequence quality was verified for every single fungal species under visualization in the SnapGene® Viewer 3.2 (SnapGene software, from GSL Biotech; available at snapgene. com), where automatically deduced sequences were also manually corrected if necessary. The single sequences were prepared for further analysis with either GeneDoc 2.7 (Nicholas and Nicholas, 1997) or MEGA 7.0 (Sudhir et al., 2016). The maximum likelihood analyses were done with the IQ-Tree-Web-Server (Nguyen et al., 2015; Trifinopoulos et al., 2016) using the ultrafast bootstrap analysis (Minh et al., 2013). The generalised time reversible (GTR) model was chosen as substitution model, using the web server FindModel (Posada and Crandall, 1998) and combined with a FreeRate heterogeneity (rate categories = 6) and 10,000 bootstrap alignments with the SH-aLRT

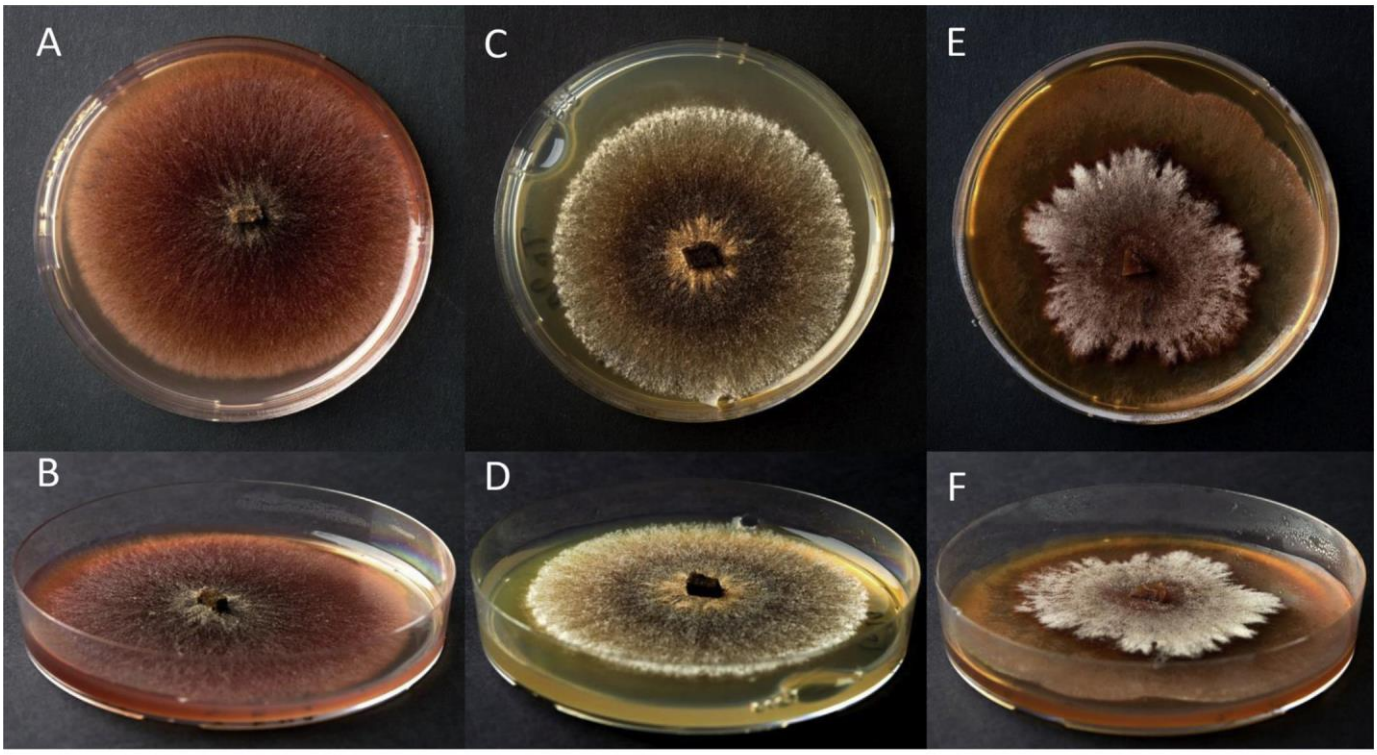

Fig. 1. Representative images of the *Phialophoropsis* species isolated from: (A, B) *Trypodendron domesticum* (*Phialophoropsis* sp.), (C, D) *Trypodendron signatum* (*Phialophoropsis* sp.) and (E, F) *Trypodendron lineatum* (*Phialophoropsis ferruginea*).

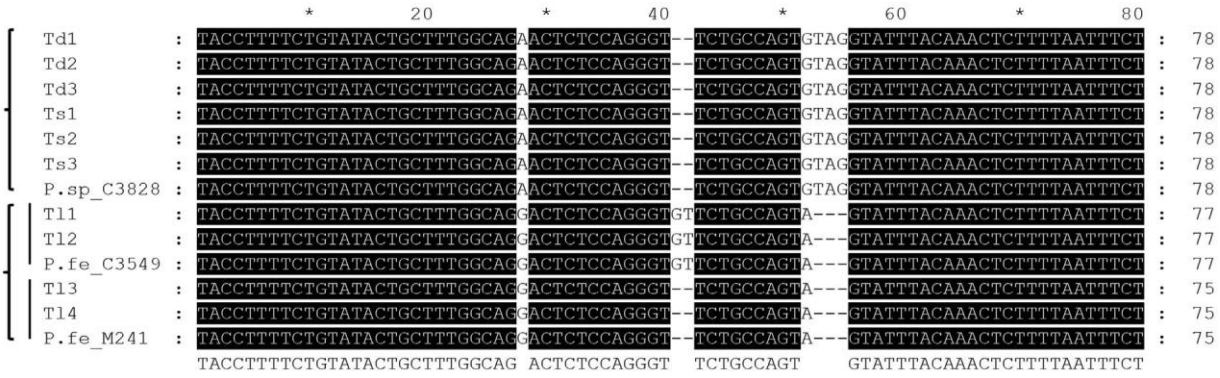

Fig. 2. Alignment of representative fungal ITS-sequences per beetle, Td1, 2, 3 = isolated out of *T. domesticum*; Ts1, 2, 3 = isolated out of *T. signatum;* Tl1, 2, 3, 4 = isolated out of *T. lineatum*. Three GenBank reference sequences were used: P. sp_C3828 = *Phialophoropsis* sp. (C3828, **MF399188.1**); P. fe_C3549 = *Phialophoropsis ferruginea* (C3549, **MF399187.1**); P. fe_M241 = *Phialophoropsis ferruginea* (M241, **MF399197.1**). The figure shows the variation within the fungal ITS-Sequences for Td 1–3, Ts 1–3 and P. sp_C3828 (… A … – … GTAG …), for Tl1, 2 and P. fe_C3549 (… G … GT … A– …) and for Tl3, 4 and P. fe_M241 (… G … – … A– …). Only a partial alignment is shown; the remaining sequence was completely identical for all examined fungal isolates.

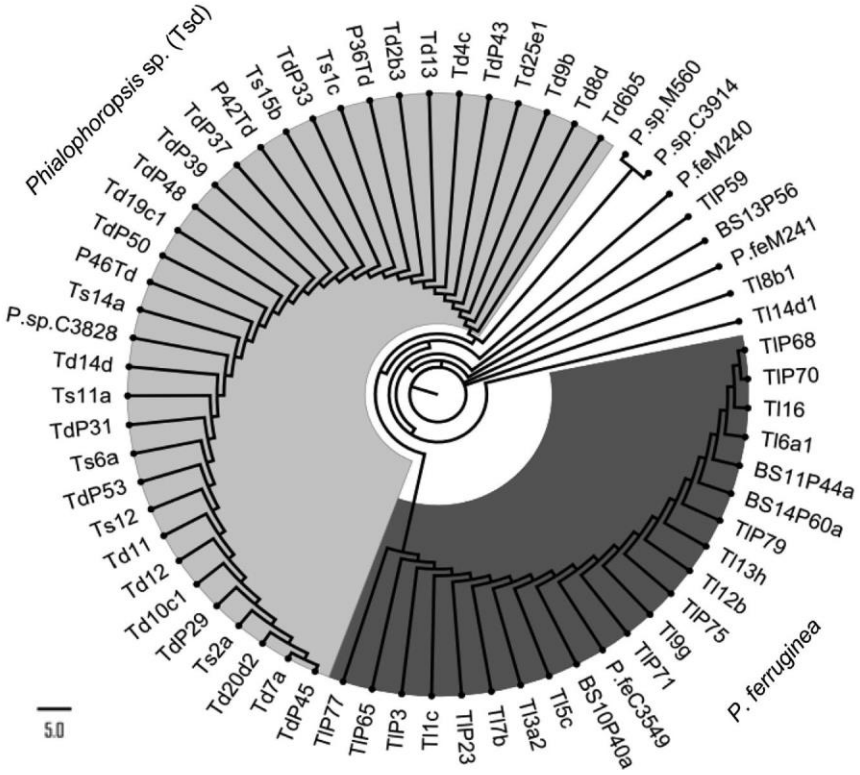

Fig. 3. Phylogenetic placement of all fungal ITS sequences obtained within this study (N = 59). IDs beginning with Ts (*Trypodendron signatum*), Td (*T. domesticum*) or Tl (*T. lineatum*) were isolated directly out of the beetles mycetangia or prothorax. IDs including a P indicate an isolate out of the *Trypodendron* lab culture, and BS indicates an isolate directly obtained out of the fresh breeding systems from the Bavarian Forest National Park. Utilized GenBank sequences: *Phialophoropsis* sp. (C3828, **MF399188.1**; M560, **MF399196.1** and C3914, **MF399189.1**). *Phialophoropsis ferruginea* (C3549, **MF399187.1**; M240, **KR611328.1** and M241, **MF399197.1**).

branch test (replicates = 10,000) as the working conditions. The tree visualizations were done with FigTree (http://tree.bio.ed.ac.uk/software/figtree).

2.6. Enzymatic profile

The physiological characterization of the wood-decomposing potential was performed with enzyme assays from secreted proteins. To obtain enough fungal biomass, pre-cultures for every fungus were grown in a liquid YEME medium (modified with 0.67% malt-extract and 0.5% peptone, 0.3% yeast-extract, 1% D-glucose, pH 5.5, at RT) in single Petri dishes. Since it was noticed that the fungal isolates showed only weak growth in the liquid cultures compared to solid media, autoclaved sandwich paper was used as a floating artificial growth surface (Antje Labes, FH Flensburg, personal communication). To that end, an agar piece with mycelium was placed on an autoclaved piece of a sandwich paper (approximately 3 × 3 cm). The pre-cultures were then transferred to a yeast nitrogen base medium (YNB, Sigma-Aldrich) containing 1% glucose for 7 d and finally switched to YNB medium with 1.5% finely milled

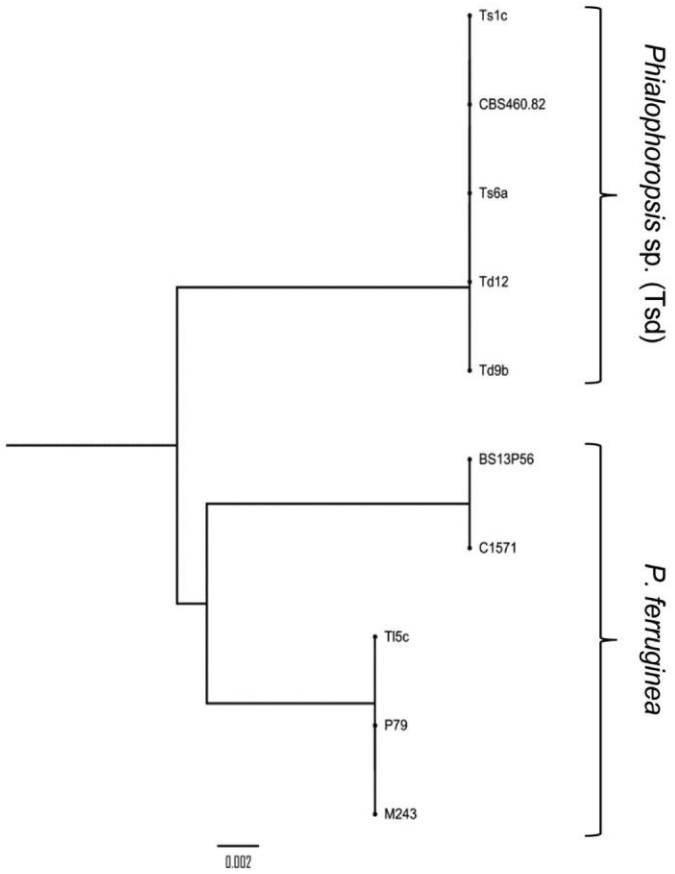

Fig. 4. Phylogenetic placement of seven representative fungal TEF1α sequences. Two isolates of *Phialophoropsis* sp. out of *T. signatum* (Ts1c, Ts6a) and two isolates of *Phialophoropsis* sp. out of *T. domesticum* (Td12, Td9b) form one cluster, including a *Phialophoropsis* sp. sequence from GenBank (CBS 460.82, **KT318388.1**). Sequence BS13P56 clusters with *P. ferruginea* sequence C1571 (HM569641.1) and Tl5c/P79 with sequence M243 (*P. ferruginea*, **KT318387.1**).

sawdust (2 x *Phialophoropsis* sp. (Td10c1, Ts11a), 2 x *P. ferruginea* (Tl5c, Tl7b), with three replicates per fungus) and 50 μg/ml streptomycin sulfate for 9 d. Due to the ecology of the beetles, spruce-sawdust (*Picea* sp.) was used for the *P. ferruginea* samples and sawdust from beech (*F. sylvatica*) for the two *Phialophoropsis* sp. (Tsd) isolates. The ITS of the final fungal biomass was checked by sequencing to exclude the probability of a contamination. Supernatants (300 μl) were taken on day 0, 3, 5 and 9, centrifuged (7.200×*g*) to remove remaining fungal biomass within the supernatant and stored at 4 °C. All enzyme assays were done at the same day under equal conditions. For every assay, 50 μl substrate (AZCL-Galactomannan, S-AXBL-Xylan (birchwood) and S-ACMCL-Glucan; Megazyme, Ireland) was mixed with 1 M sodium acetate (NaOAc), pH 5.0 (12.5 μl for endo-xylanase and endo-glucanase, 50 μl for endo-mannanase) and culture supernatant (37.5 μl for endo-xylanase and endo-glucanase, 50 μl for endo-mannanase). Additionally, two blanks with deionized water were measured for every assay. An incubation time of 60 min with an assay temperature of 40 °C using a water bath was used, followed by different precipitation solutions for endo-glucanase (24.1 g NaOAc (anhydrous) + 4 g ZnAc with 150 ml ddH_2O and 800 ml 96% EtOH, pH 5.0; 250 μl per sample), endo-xylanase (250 μl 96% EtOH) and endo-mannanase (500 μl 2% Tris-buffer). After a spin for 2 min at 1.800×*g* (10 min at 2000×*g* for endo-mannanase), 200 μl of each sample supernatant were transferred to a 96-well plate, and measured at 590 nm in a Microplate Reader (Infinite 200 PRO; Tecan, Germany). The visualization was done with R-Studio (Version 3.1.2), using *ggPlot* and the packages *lattice* (Sarkar, 2008), *ggplot2* (Wickham, 2016) and *plyr* (Wickham, 2011).

As a further investigation of the wood-degrading potential, two *Phialophoropsis* fungi (1× *Phialophoropsis* sp. (Ts11a), 1× *P. ferruginea* (Tl5c)) were grown on a solid 1× Vogel's medium with 2% agar and 0.5% of either xylan (from beechwood, Sigma-Aldrich, Germany), xylose (Sigma-Aldrich, Germany) or D-glucose (Roth, Germany) with four replicates per fungi and carbon source. In each case, 50 μg/ml streptomycin sulfate salt were added. The fungal growth on each Petri dish was observed for 14 d under room conditions, while the radial fungal growth was measured for each sample and sugar at d 14. A significant preference for one of the available carbon sources was tested for each individual fungus using R and a one-way-ANOVA followed by a *post hoc* TukeyHSD test. The results were plotted with *ggPlot*, utilizing the packages *lattice* (Sarkar, 2008), *ggplot2* (Wickham, 2016) and *plyr* (Wickham, 2011).

2.7. Variability within the genus Phialophoropsis

All fungal ambrosia isolates were divided into 34 different morphotypes, which were grown on a YEMA 2 medium in the following manner: First for 7 d under room conditions (about 25 °C), followed by 14 d in a cooling room (3–6 °C) and finally again 7 d under room conditions. For documentation, photos (Panasonic TZ7 12×, mode = intelligent automatic) of all phenotypes were taken at the end of each step.

3. Results

3.1. Associated fungi

In total, 81 fungi were isolated from three different sources: (1) trapped *T. lineatum*, *T. domesticum* and *T. signatum* beetles (from mycetangium, prothorax and gut), (2) active breeding systems cut from recently felled trees, and (3) *Trypodendron*-inoculated artificial rearing media (see Methods). Out of 59 beetles/galleries, we

Table 1
Utilized fungal references with the specimen identification number (ID) and the corresponding GenBank accessions for ITS and TEF1α.

Annotation	ID	ITS	TEF1 α	Associated ambrosia beetle	References
Phialophoropsis sp.	M560	**MF399196.1**		*Trypodendron signatum*	Mayers and Harrington, unpublished
Phialophoropsis sp.	C3828	**MF399188.1**		*Trypodendron domesticum*	Mayers and Harrington, unpublished
Phialophoropsis sp.	C3914	**MF399189.1**		*Indocryphalus pubipennis*	Mayers and Harrington, unpublished
Phialophoropsis ferruginea	C3549	**MF399187.1**		*Trypodendron lineatum*	Mayers and Harrington, unpublished
Phialophoropsis ferruginea	M240	**KR611328.1**		*Trypodendron lineatum*	Mayers et al., 2015
Phialophoropsis ferruginea	M241	**MF399197.1**		*Trypodendron lineatum*	Mayers and Harrington, unpublished
Ambrosiella ferruginea	C1571		HM569641.1		Harrington, unpublished
Phialophoropsis ferruginea	M243		**KT318387.1**	*Trypodendron lineatum*	Mayers et al., 2015
Phialophoropsis sp.	CBS 460.82		**KT318388.1**	*Trypodendron domesticum*	Mayers et al., 2015

identified 33 ambrosia fungi from *T. domesticum*, 36 from *T. lineatum* and 12 from *T. signatum* (see Fig. 1 for photographs from representative isolates). No ambrosia fungi were isolated out of 24 beetles (total 83). All sequenced fungi were identified as species in the ambrosia fungal genus *Phialophoropsis* (Table 2). *P. ferruginea* was isolated from more than 77% of investigated beetles, whereas *Phialophoropsis* sp. (Tsd) was prevalent in nearly 70% of the examined *T. domesticum* beetles and in 70% of the *T. signatum* beetles (Table 2). The obtained ITS sequences of the *T. domesticum* and *T. signatum* isolates were found to be completely identical with a *Phialophoropsis* sp. sequence from GenBank (C3828, MF399188.1) (Figs. 2 and 3) and were designated "*Phialophoropsis* sp. (Tsd)" to enable a better distinction. Fungal isolates of *T. lineatum* were clearly assigned as *P. ferruginea*, but showed some variation within one to three locations in the ITS sequences within the obtained sequences and in comparison to the GenBank entries. Most of the received fungal ITS sequences from *T. lineatum* were identical with the *P. ferruginea* sequence C3549 from GenBank (MF399187.1). The remaining *T. lineatum* isolates were more similar to the *P. ferruginea* sequence M241 (MF399197.1). The ITS-phylogeny of all fungal isolates (Fig. 3) was in line with the results of the alignments (Fig. 2).

The TEF1α region was analyzed for seven *Phialophoropsis* isolates: 2× *Phialophoropsis* sp. (Td12, Td9b) from *T. domesticum*, 2× *Phialophoropsis* sp. (Ts1c, Ts6a) from *T. signatum* and 3× *P. ferruginea* from *T. lineatum* (BS13P56, Tl5c, TlP79), representing the most common morphotypes of each fungus (Fig. 4). The result was highly similar to the ITS phylogeny, with the CBS 460.82 reference (*Phialophoropsis* sp.; KT318388.1) matching with all of the *T. signatum* and *T. domesticum* isolate sequences, while the *T. lineatum* isolates were split again, matching either to reference M243 (KT318387.1) or C1571 (HM569641.1) (both designated *P. ferruginea*).

In summary, our phylogenetic analysis confirmed the presence of a putatively new fungal species (*Phialophoropsis* sp. (Tsd)) as the fungal symbiont of *T. domesticum* and *T. signatum*. Moreover, a so far undescribed variation within the *P. ferruginea* sequences was detected, possibly indicating an unknown kind of divergence.

In addition to the ambrosia fungi, two yeasts were isolated and identified by ITS sequencing (1× *Candida trypodendroni* and 1× *Cystobasidium laryngis*). By visual inspection, *C. trypodendroni*-like yeasts were found in about 70% of all three studied *Trypodendron* species, while *C. laryngis* was found in one *T. signatum* and two *T. domesticum* beetles.

3.2. Physiological characterization

3.2.1. Enzymatic activity

With wood as the main carbon-source for the growing ambrosia fungi, our enzymatic characterization targeted endo-β-1,4-glucanase, endo-β-1,4-xylanase, and endo-β-1,4-mannanase activities. These were chosen as being representative for the degradation potential of the fungi for the main wood cell wall polysaccharides: xylan and mannan as the major hemicelluloses as well as cellulose. Four *Phialophoropsis* isolates (2× *P. ferruginea* (Tl5c, Tl7b), 2× *Phialophoropsis* sp. (Tsd) (Td10c1, Ts11a)) were chosen for the investigation as representatives for the other obtained fungal isolates.

Even though a cross-comparison between assays is difficult due to the varying recalcitrance of the used substrates, robust activities were measured for endo-glucanase (Fig. 5 A) and endo-xylanase (Fig. 5 B), while endo-mannanase activity could not be detected and is therefore not depicted. Production of endo-xylanase over the observed period was similar for *Phialophoropsis* sp. (Tsd) and *P. ferruginea* isolates, and activity slowly increased over time up to a relatively high level. Also the cellulolytic activity was prominent for both *Phialophoropsis* spp. but was observed to increase more quickly for *P. ferruginea* than for *Phialophoropsis* sp. (Tsd) (highest activity at 5 d *vs*. 9 d; Fig. 5 A).

The ability of the tested ambrosia species to efficiently degrade xylan could be verified for both species by using an artificial agar-based growth medium with xylan as only C-source. After 14 d of incubation, the radial growth of *P. ferruginea* and *Phialophoropsis* sp. (Tsd) was significantly faster on xylan than on glucose (Fig. 5C). Additionally, the radial growth of *P. ferruginea* on xylose was also significantly higher than on glucose.

3.2.2. Morphological variability

Inspired by an investigation made more than 70 y ago (Leach et al., 1940), noticing high morphological variation within the *Phialophoropsis* spp., a growth experiment including a temperature shift was carried out (Suppl. Fig. 2). As observed before, even identical fungal isolates grown under identical conditions displayed immense variability in coloration, regarding the discoloration of the fungal mycelia and the secretion of coloring substances into the media. This effect was most prominent after 14 d in the cooling chamber (Suppl. Fig. 2, 28d), when coloration became most intense.

4. Discussion

The fungal ambrosia genus *Phialophoropsis* has long been known to be associated with *Trypodendron* bark beetles (Batra, 1967; Francke-Grosmann, 1967). Our study finally confirmed high species specificity using large sample sizes (59 successful isolations from a total amount of 83 studied beetles); *T. lineatum* and *P. ferruginea* are tightly associated (>77% of beetles; Table 2), whereas *T. domesticum* (70%) and *T. signatum* (70%) share the same *Phialophoropsis* sp. (Tsd)) symbiont.

Previously, *P. ferruginea* was thought to be associated with all three *T. lineatum*, *T. domesticum* and *T. signatum* (Funk, 1965; Batra,

Table 2
Prevalence of the *Phialophoropsis* species isolations out of the three *Trypodendron* beetle species subdivided by way of isolation (directly = out of prothorax or mycetangium). The breeding systems are not counted to the total amount of beetles.

Beetle species	N of tested beetles	Fungal isolate	Fungal prevalence [%]	Counts
T. domesticum	**total = 39**	*Phialophoropsis* sp. (Tsd)	**69.23**	27
	directly = 25		56	14
	lab-culture = 14		92.86	13
	breeding-systems = 7		0	0
T. lineatum	**total = 27**	*P. ferruginea*	**77.8**	21
	directly = 15		73.3	11
	lab-culture = 12		83.4	10
	breeding-systems = 7		57.14	4
T. signatum	**total = 10**	*Phialophoropsis* sp. (Tsd)	**70**	7
	directly = 10		70	7

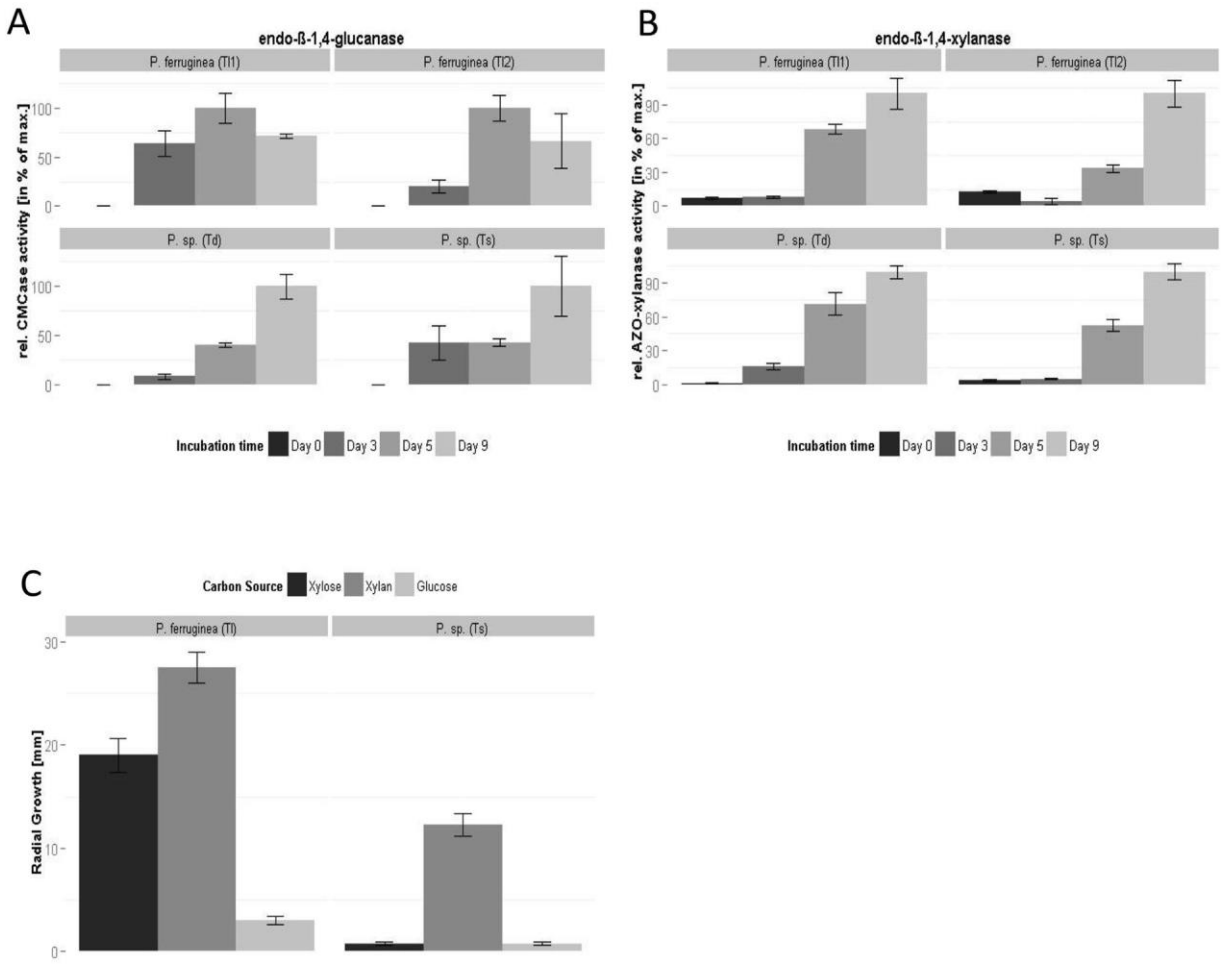

Fig. 5. Enzymatic activities on wood and wood-derived polysaccharides. (A) = endo-β-1,4-glucanase activity, (B) = endo-β-1,4-xylanase activity on wood meal. Tl1 and Tl2 = Fungal isolate out of two different *T. lineatum* beetles. Td = Fungal isolate (*P. ferruginea*) out of *T. domesticum*, Ts = Fungal isolate out of *T. signatum*, both *Phialophoropsis* sp., N = 3 per fungus; Relative values were used which were normalized (100%) for each of the max. activities. Means with their standard errors are given. (C) Barplot of the radial fungal growth in dependence of the available carbon source (xylan, xylose and glucose) of two representative *Phialophoropsis* fungi (N = 4 per fungus; *Phialophoropsis* sp. (Tsd) = P. sp. (Ts) = Ts11a and *Phialophoropsis ferruginea* = *P. ferruginea* = Tl5c). Tl indicates an isolation out of *Trypodendron lineatum* and Ts out of *Trypodendron signatum*. Fungal samples growing on xylan showed a significantly higher radial growth (***P.sp.* (Ts)**: Xylan − Glucose, $p < 0.001$, Xylan − Xylose, $p < 0.001$; ***P. ferruginea* (Tl)**: Xylan − Glucose, $p = 0.0036$, Xylan − Xylose, $p < 0.001$, Xylose − Glucose, $p < 0.001$).

1967; Francke-Grosmann, 1967). However, slight differences within the ITS and TEF1α sequences as well as a morphological deviation in regard to a fungal isolate of *T. signatum* had already been noticed in earlier studies (Nakashima et al., 1992; Mayers et al., 2015). In the current study, nucleotide alignments finally confirmed a slight but constant deviation between the ITS sequences of the *T. lineatum* and the *T. domesticum/signatum* isolates (Fig. 2), separating these groups also in a phylogenetic analysis (Fig. 3). A highly analogous result could be drawn from analysis of the TEF1α locus (Fig. 4), finally allowing us to distinguish between the fungal symbionts of *T. lineatum* and *T. domesticum/signatum* with ITS and TEF1α as marker genes.

While all *T. domesticum* and *T. signatum* isolates were found to be completely identical on a molecular basis, sequence deviation was surprisingly detected within the *T. lineatum* isolates: The *T. lineatum* isolate BS13P56 for example differed from the majority of isolates both in the TEF1α (Fig. 4) and ITS alignments (together with three other *T. lineatum* isolates; Fig. 3). With a total of four fungal isolates in this study and several highly similar entries in GenBank (M240, **KR611328.1**; M241, **MF399197.1**; C1571, **HM569641.1**), this does not appear to be a unique observation, but might represent a real genetic variation within this fungal species. However, we do not have sufficient evidence to declare a new species for *P. ferruginea* so far. To safely confirm a novel *Phialophoropsis* species, the sequencing of further genetic markers would be required. In the future, it will be interesting to investigate whether this variation in *P. ferruginea* is also reflected in genetic differentiation in the beetles. There might be a fourth cryptic *Trypodendron* species co-occurring in the same habitat. *Trypodendron laeve*, is a possible candidate, because it is very difficult to discern morphologically from *T. lineatum* and has been found in the study area. *T. laeve* also breeds in conifers and its fungus has currently not been identified.

Regarding the *Phialophoropsis* GenBank entries, it should be mentioned that the *P. ferruginea* sequence CBS 460.82 (KC305146.1) was identical to the *Phialophoropsis* sp. (Tsd) sequence and should thus probably be re-annotated. On the other hand, two *Phialophoropsis* sp. ITS GenBank sequences were identical with the *P. ferruginea* sequence from this study and should also be re-annotated (**MF399194.1**, M594; **MF399190.1**, C3386). Moreover, some GenBank sequences (MF399193.1, M593; **MF399191.1**, C3404;

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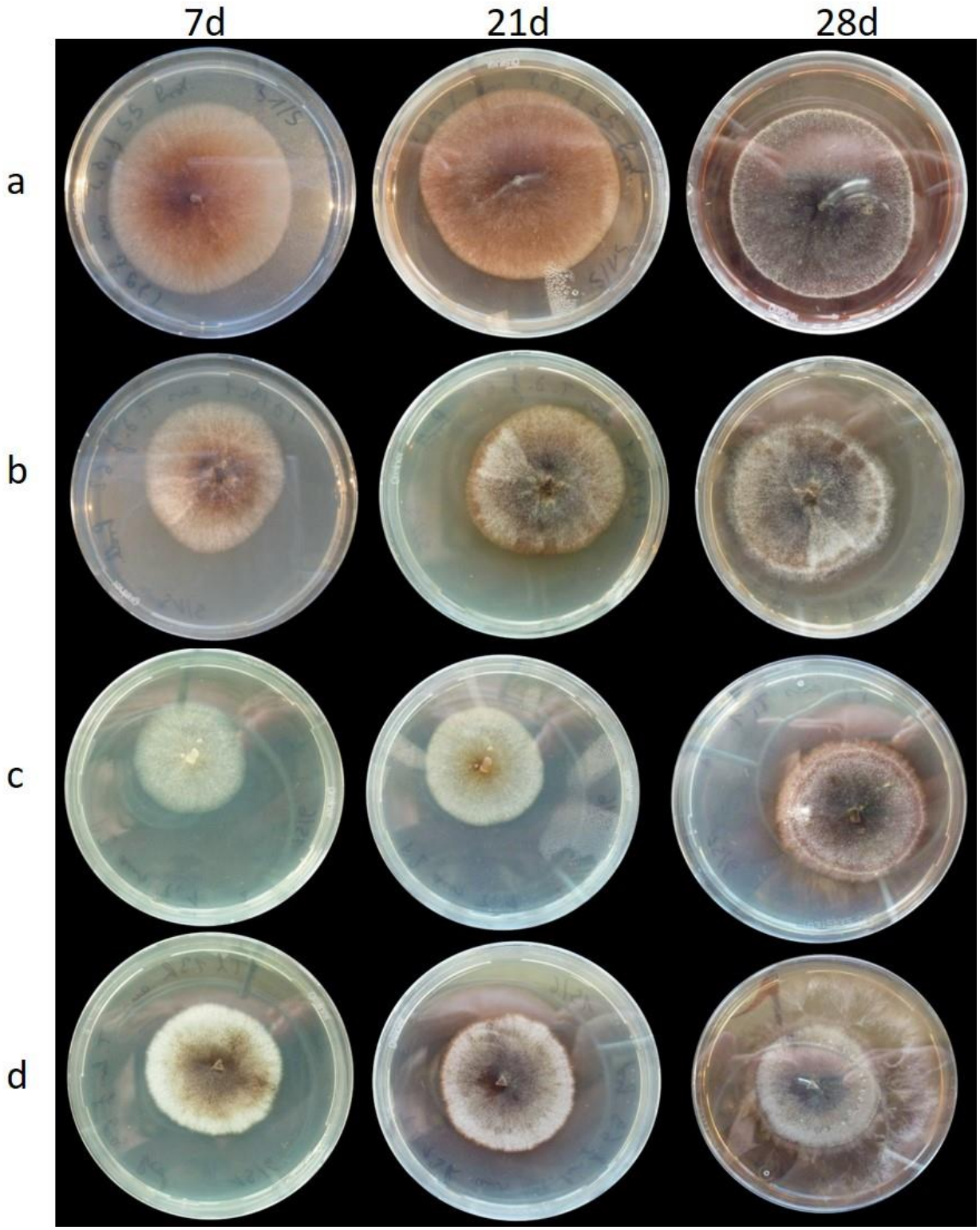
Annex

Suppl. Figure 1: Pheromone baited trap on beech (a) and on spruce (b) and tree stumps (c = spruce, d = beech) used for cutting out active breeding systems, (a-d = located in the Bavarian Forest National Park). e = gallery of *Trypodendron domesticum* in beech, f = gallery and larvae of *T. domesticum* in beech, g = gallery of *T. domesticum* in beech, microscopy (10×), h = adult of *T. lineatum* in spruce, i = gallery of *T. lineatum* in spruce, microscopy (10×), j = gallery of *T. lineatum* in artificial rearing-medium, microscopy (5×).



Suppl. Figure 2: Photographs of fungal phenotypes over time in temperature switch assay. (a & b) = *Phialophoropsis* sp. (Tsd), (c & d) = *Phialophoropsis ferruginea*. Each row is showing the same fungal isolate (a, b, c, or d) after 7 days of growth at room condition (7d), followed by 14 days at 3-6°C (21d) and again 7 days at room condition (28d). Overall, the fungi were incubated for 28 days on YEMA 2 medium.

Chapter 2

Lehenberger M, Thalhammer M, J Benz P and Biedermann PHW. (2019). – Take good care of my fungi – Fungus-spore carrying organs in *Trypodendron* ambrosia beetles. *Mitteilungen der deutschen Gesellschaft für allgemeine und angewandte Entomologie* 22

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– Take good care of my fungi – Fungus-spore carrying organs in *Tryopodendron* ambrosia beetles

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Abstract: Pilz-züchtende Ambrosia-Käfer (Coleoptera: Scolytinae) sind durch ihre Symbiose mit mutualistischen Pilzen gekennzeichnet, welche ihnen als essentielle Nahrungsquelle dienen. Die Käfer kultivieren aktiv einen oder mehrere ihrer Nahrungspilze (Ambrosia-Pilze) in ihren Tunnelsystemen, die sie im Xylem toter oder stark geschwächter Bäume anlegen. Die Ambrosia-Pilze besiedeln die Brutsysteme und bilden dabei asexuelle Fruchtkörper an den Tunnelwänden, welche die Hauptnahrungsquelle der Käfer und Larven sind. Zur Übertragung der Ambrosia-Pilzsporen aus dem Geburtsnest in ein neu gegründetes Nest besitzen die Weibchen paarweise angeordnete, ektodermale Organe – sogenannte Mycetangien. Da sich die Pilzzucht innerhalb der Scolytinae mehrmals unabhängig entwickelte, unterscheiden sich die Käfer stark hinsichtlich Lage und Struktur der Mycetangien. Für viele heimische Ambrosia-Käfer sind die Morphologie und Lage dieser Strukturen noch nicht untersucht.
In dieser Studie analysierten wir erstmalig die Mycetangien von *Tryopodendron laeve* Eggers, einem relativ seltenen Europäischen Ambrosia-Käfer. Mittels zerstörungsfreier Röntgentomographie (µCT) entdeckten wir ein gepaartes, röhrenförmiges, pleural-prothorakales Mycetangium, welches den bereits bekannten Strukturen anderer *Trypodendron*-Arten ähnelt. Die Morphologie des gefundenen Mycetangiums wurde mit den bereits beschriebenen Organen von *Tryopodendron domesticum*, *T. signatum* und *T. lineatum* verglichen. Des Weiteren untersuchten wir mit Hilfe der Rasterelektronenmikroskopie (REM) vermutliche tracheale Strukturen in der Nähe der Mycetangien. Diese Tracheen, die sowohl bei Weibchen als auch bei den Männchen vorhanden sind, könnten zur Belüftung der Pilzsporenorgane dienen.

Keywords: Scolytidae, *Trypodendron*, mycetangium, mycangium, Ascomycota, *Phialophoropsis*

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Introduction

The bark beetle genus *Trypodendron* (Coleoptera: Curculionidae, Scolytidae) includes 14 species worldwide, from which at least four are common inhabitants of European forests. The European species, *Trypodendron lineatum* (Olivier 1795), *T. domesticum* (Linnaeus 1758), *T. signatum* (Fabricius 1787) and *T. leave* (Eggers 1939) are classified as fungus-farming ambrosia beetles (Francke-Grosmann 1956, Bussler & Schmidt 2008). They bore tunnels in the wood of recently dead or weakened trees and cultivate species-specific fungi of the genus *Phialophoropsis* (Ascomycota: Ceratocystidaceae) within their galleries (Francke-Grosmann 1956, 1966, Mayers & al. 2015, Lehenberger & al. 2019). As the *Trypodendron* species overwinter either within their galleries (*T. domesticum*, Parini & Petercord 2006), the organic layer on the ground (*T. lineatum*, Oranen 2013) or under the bark of their host trees

(*T. signatum* & *T. laeve*, Schwenke 1974, Bussler & Schmidt 2008) they depend on species-specific, pairwise arranged spore-carrying organs to transport and store their fungal symbionts during hibernation and for transmission into a newly excavated breeding gallery in spring. These organs have evolved independently several times within the Scolytidae (Six 2003) and have been termed mycetangia (Sg. mycetangium) (Giese 1967) or mycangia (Sg. mycangium) (Batra 1963). Mycetangia typically are invaginations of the integument, lined with glands or secretory cells (Batra 1963, Levieux & al. 1991).

Although the ecology is well studied for the European *Trypodendron* species (Francke-Grosmann 1956, Nakashima & al. 1992, Mayers & al. 2015, Lehenberger & al. 2019), only schematic figures from the mycetangia were provided up to date (Francke-Grosmann 1956). Mycetangia in *Trypodendron* have been described as paired, tubular, pleural-prothoracic organs (Francke-Grosmann 1956, Batra 1967, Mayers & al. 2015). The structure is similar for at least three of the European species; slight differences were only noticed regarding the size and shape (Francke-Grosmann 1956; Fig. 1 and 2). Only female *Trypodendron* species are known to carry the mycetangia (Francke-Grosmann 1956). No investigation has so far examined the mycetangial structures of *T. laeve*.

Here, we examined the mycetangial structure of an adult female *T. laeve*, using non-destructive X-ray tomography (µCT). Moreover, we investigated putative tracheal structures close to the beetles´ mycetangia in both female and male *T. domesticum, T. signatum* and *T. lineatum* using scanning-electron-microscopy (SEM).

Materials and Methods

Beetle collection: Beetles were collected at the end of March 2017 in the Bavarian Forest National Park (Bavaria, Grafenau). We used pheromone-baited traps ("Lineatin Kombi" by Witasek). Subsequently, caught beetles were individually stored in 1.5 ml Eppendorf Tubes filled with a piece of damp tissue paper. During sampling, beetles were stored at 4 °C. Beetles were finally identified in Freising (TU Munich) under lab-conditions (Grüne 1979, Bussler & Schmidt 2008) and stored alive (3–6°C) until usage.

Morphological description: For Scanning-Electron-Microscopy (JEOL/EO, Version 1.000), we first prepared one male and one female of each beetle species (*T. domesticum*, *T. lineatum, T. signatum*). Therefore, we made putative tracheal structures visible by carefully pushing the pronotum away from the mesonotum (similar to Bateman & al. 2016), using minute pins (Bioform). Additionally, we investigated the prothorax of some beetles without removing their heads. In the latter case, we just cut off the mesothorax and the front legs. Subsequently, beetles were air-dried for at least 24 h.

For the micro-Computed Tomography (µCT) we investigated only one female *T. leave* specimen. The beetle was dehydrated by an ethanol (absolut) dehydration series (30 %, 50 %, 70 %, 80 %, 90 %; each step for 20 min) and finally stored within 100 % EtOH, containing 1% iodine (Alba-Tercedor & Hunter 2014). Afterwards, the beetle was air-dried for 24 h within a sterile hood. The measurements were implemented at the chair of process systems engineering (TU Munich, Freising). Here we used the XCT-1600 HR V1.1 computer tomograph (Matri-Technologies, software: MatriX MIPS-NDT) with a range of 1 µm and 2000 projections. The data were analyzed and visualized (images and videos) using the software MAVI V1.4.1.

Results and Discussion

The µCT examination of a female *T. laeve* revealed a paired, tubular, pleural-prothoracic mycetangium (see Fig. 3a–e). Therefore, we can conclude that all four European *Trypodendron* species have mycetangial structures with similar morphologies (see Fig. 1 and 2; Francke-Grosmann 1956, Batra 1967, Mayers & al. 2015). However, the mycetangium of *T. laeve* is more similar to the one from *T. lineatum* than to those of *T. domesticum* or *T. signatum* (Francke-Grosmann 1956). It is likely that we detected a possible opening (exit or entrance) of *T. laeve´*s mycetangium located behind the procoxa. This assumption was made due to a visible bulge in the lower part of the mycetangium, ending near the procoxa (see Fig. 3d). It seems to directly end in the beetle´s inner cuticula. Similar structures have not been described by Franke-Grosmann (1956) for any of the other *Trypodendron* species. However, such variations within a scolytine genus would not be unusual. The presence and type of mycetangia can vary within a genus, though mycetangia types are often genus-specific (Six 2003). It is noteworthy that the µCT-preparation

method could have influenced the beetle´s inner structures, however. Through the storage of the beetles within ethanol and the drying before the measurement, these structures might have been affected. Even though the beetles were slowly dehydrated, a slight change of the inner structures cannot be completely excluded.

The putative tracheal structures were found to be similar for all of the three investigated *Trypodendron* species and were observed in both females and males (see Fig. 3 f & g). Slight differences were only noticed regarding the size, shape and location. They all showed a conspicuous large, sclerotized surface with thin and fine edges, which are covered with fine eyelash-like hairs. Furthermore, all tracheal structures were located within the lower part of the prothorax. These structures may serve the aeration of the beetle´s mycetangia, but this hypothesis needs to be investigated in the future in more detail.

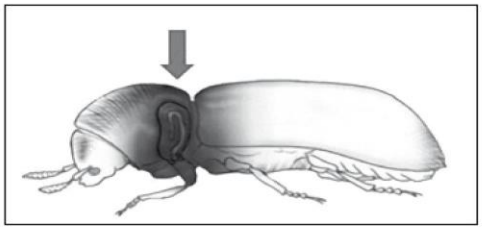

Fig 1: Location of the prothoracid mycetangium within Tryopodendron females.

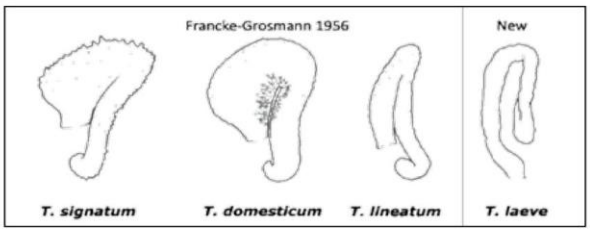

Fig 2: Drawings of mycetangia of Trypodendron spp. (modified from FRANCKE-GROSMANN (1956)) and the newly described mycetangium of T. leave.

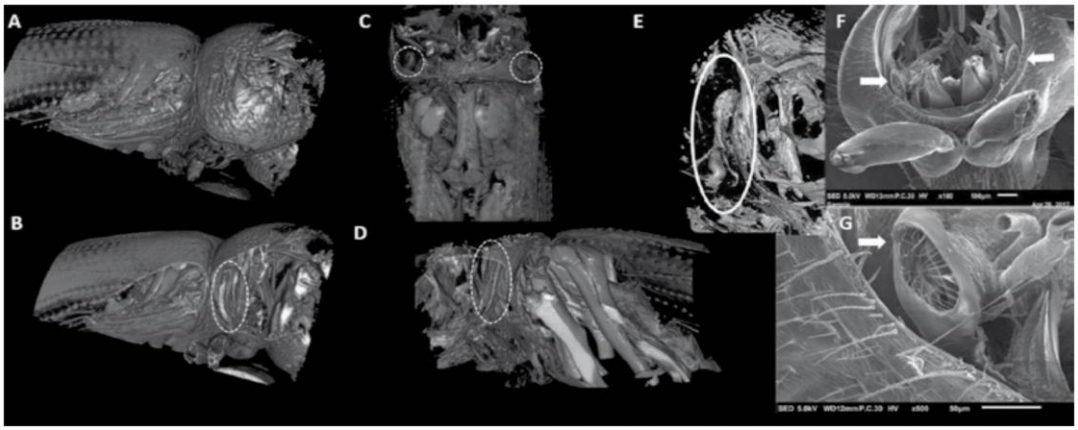

Fig 3: µCT images of *T. laeve*. A: Entire beetle, B: Right side view, C: View from above D: Left side view, notice the bulge at the lower end, E: Complete Mycetangium; Mycetangial structures are marked with a white circle; F & G: Putative tracheal structure of *T. domesticum*. Structures are indicated by arrows.

Conclusions

The µCT-examination of the ambrosia beetle *T. laeve* revealed a paired, pleural-prothoracic mycetangium. This kind of mycetangium is typical for the whole scolytine ambrosia beetle genus *Trypodendron*. Additionally, using scanning-electron-microscopy, we investigated putative tracheal structures close to the beetles´ mycetangia and provide the first SEM images of these structures in T. domesticum, T. signatum and T. lineatum.

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Chapter 3

Lehenberger M, Benkert M and Biedermann PHW. (2021). Ethanol-Enriched Substrate Facilitates Ambrosia Beetle Fungi but Inhibits Their Pathogens and Fungal Symbionts of Bark Beetles. *Frontiers in Microbiology*, 11, 3487.

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Ethanol-Enriched Substrate Facilitates Ambrosia Beetle Fungi, but Inhibits Their Pathogens and Fungal Symbionts of Bark Beetles

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Bark beetles (*sensu lato*) colonize woody tissues like phloem or xylem and are associated with a broad range of micro-organisms. Specific fungi in the ascomycete orders Hypocreales, Microascales and Ophistomatales as well as the basidiomycete Russulales have been found to be of high importance for successful tree colonization and reproduction in many species. While fungal mutualisms are facultative for most phloem-colonizing bark beetles (*sensu stricto*), xylem-colonizing ambrosia beetles are long known to obligatorily depend on mutualistic fungi for nutrition of adults and larvae. Recently, a defensive role of fungal mutualists for their ambrosia beetle hosts was revealed: Few tested mutualists outcompeted other beetle-antagonistic fungi by their ability to produce, detoxify and metabolize ethanol, which is naturally occurring in stressed and/or dying trees that many ambrosia beetle species preferentially colonize. Here, we aim to test (i) how widespread beneficial effects of ethanol are among the independently evolved lineages of ambrosia beetle fungal mutualists and (ii) whether it is also present in common fungal symbionts of two bark beetle species (*Ips typographus*, *Dendroctonus ponderosae*) and some general fungal antagonists of bark and ambrosia beetle species. The majority of mutualistic ambrosia beetle fungi tested benefited (or at least were not harmed) by the presence of ethanol in terms of growth parameters (e.g., biomass), whereas fungal antagonists were inhibited. This confirms the competitive advantage of nutritional mutualists in the beetle's preferred, ethanol-containing host material. Even though most bark beetle fungi are found in the same phylogenetic lineages and ancestral to the ambrosia beetle (*sensu stricto*) fungi, most of them were highly negatively affected by ethanol and only a nutritional mutualist of *Dendroctonus ponderosae* benefited, however. This suggests that ethanol tolerance is a derived trait in nutritional fungal mutualists, particularly in ambrosia beetles that show cooperative farming of their fungi.

Keywords: ambrosia fungi, bark and ambrosia beetles, symbiont selection, ethanol, detoxification, *Ips typographus*

INTRODUCTION

Filamentous fungi are generally known to be common symbionts of bark and ambrosia beetles (Curculionidae: Scolytinae and Platypodinae). While many species use beetles for dissemination with apparently little benefits for their vectors (Birkemoe et al., 2018; Seibold et al., 2019) other fungi are essential for the beetles like nutritional or tree-defenses-detoxifying mutualists as well as tree-defenses-stimulating fungi (e.g., Six, 2003; Lieutier et al., 2009; Kandasamy et al., 2019; Biedermann and Vega, 2020). The most famous nutritional mutualisms are those between xylem-colonizing ambrosia beetles and various "ambrosia fungus" species in the ascomycete orders Hypocreales, Microascales and Ophiostomatales as well as phloem-colonizing *Dendroctonus* bark beetles and their basidiomycete and ascomycete mutualists in the orders Russulales and Ophiostomatales (Barras and Perry, 1972; Six and Paine, 1998; Six and Klepzig, 2004; Harrington, 2005; Kirkendall et al., 2015; Biedermann and Vega, 2020). All these are truly agricultural mutualisms, because they evolve active care of the beetles for their fungal "crops" and involve more or less species-specific partnerships. Indeed, within the majority of the ambrosia beetle – fungus relationships, the beetles are essential for the survival of their mutualistic fungi as they would be overgrown by fungal competitors if the beetles are not present (Kirkendall et al., 2015; Nuotclà et al., 2019). Up to date, these mutualisms are unique for beetles and comparable to the advanced fungi culturing systems of attine ants and fungus-farming termites (Farrell et al., 2001; Six, 2003; Müller et al., 2005; Biedermann and Vega, 2020).

The nutritional mutualisms of ambrosia beetles and certain *Dendroctonus* spp. as well as some *Ips* sp. are known for quite a while (Batra, 1963; Francke-Grosmann, 1965, 1966, 1967; Six, 2003; Vissa and Hofstetter, 2017), but more recent research shows that other bark beetle species also depend on fungal associates mainly through detoxification of insect-repelling tree-chemistry (like in the case of fungal symbionts of the European spruce bark beetle *Ips typographus*; Kandasamy et al., 2016, 2019; Wadke et al., 2016; Zhao et al., 2019). While nutritionally important fungal mutualists are typically vertically transmitted within mycetangia (i.e., fungus spore carrying and selecting organs), these organs are rather rare in non-nutritional beetle-fungus associations (Francke-Grosmann, 1956, 1967; Batra, 1963; Six, 2003; Hulcr and Stelinski, 2017; Skelton et al., 2020; Biedermann and Vega, 2020). How mutualisms are maintained in the latter case is poorly known and supposedly involves vertical transmission through the gut or the surface of the exoskeleton of the insects (Six, 2003; Harrington, 2005). Even less understood is how the beetles maintain the dominance of certain fungi within their tunnel systems and how they suppress ubiquitous competitor fungi and beetle pathogens (henceforth termed *antagonistic fungi*).

So far, three evolutionary mechanisms are known by which a host (e.g., a bark beetle) can maintain a mutualism with a beneficial symbiont (e.g., an ambrosia fungus): (i) Partner choice, in which a host is actively selecting a specific symbiont, (ii) partner fidelity, where symbionts are vertically transmitted from one host generation to the next, and (iii) the theoretical and empirically hardly studied mechanism of competition-based screening, in which environmental filters created by the host select for the preferred symbiont (Archetti et al., 2011; Scheuring and Yu, 2012; Foster et al., 2017). Environmental screening of mutualistic ambrosia fungi utilizing ethanol within woody substrate (that most ambrosia beetles preferentially colonize) has been recently discovered by Ranger et al. (2018). These authors show that ethanol strengthens the competitive ability of mutualistic ambrosia fungi over other fungal antagonists, because ambrosia fungi are able to detoxify ethanol and use it as a carbon source, whereas the antagonists are strongly inhibited in their growth by even small amounts of ethanol, which is typically an antimicrobial compound (McGovern et al., 2004; Tunc et al., 2007). Moreover, mutualistic ambrosia beetle fungi are known to produce ethanol and other alcohols themselves (Kuhns et al., 2014; Kandasamy et al., 2016), giving them the possibility to enrich the colonized woody substrate with ethanol and thus maintain their dominance even after the production by the dying plant cells ceases (Kimmerer and Kozlowski, 1982). The ethanol production of ambrosia fungi can thus be compared with other similar defensive mechanisms used by other microorganisms to protect themselves and their animal hosts (Cardoza et al., 2006; Scott et al., 2008; Six, 2013; Flórez et al., 2015; Ranger et al., 2018).

Ethanol generally plays a crucial role for the attraction of ambrosia beetles (i.e., it is a kairomone) as it allows them to detect suitable hosts like stressed or recently dead trees (Graham, 1968; Kühnholz et al., 2001; Ranger et al., 2015). In fact, ethanol is not solely present in stressed trees but is commonly found also in healthy trees within both xylem and phloem (Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1991; Kozlowski, 1992; Kelsey et al., 2014). Its content is known to increase as soon as the tree is stressed (e.g., flood stress, mechanical damage) (Kimmerer and Kozlowski, 1982; MacDonald and Kimmerer, 1991; Ranger et al., 2013) and is thereby functioning as a cue for ambrosia beetles to recognize defense deficient trees. The phloem-colonizing bark beetles, which are the evolutionary ancestors of ambrosia beetles, still prefer similarly deficient host trees. On the contrary, however, they usually use tree volatiles and/or aggregation pheromones (produced *de novo*) other than ethanol to detect their preferred hosts (Vité et al., 1972; Wood, 1982; Kirkendall et al., 2015; Kandasamy et al., 2016; Biedermann et al., 2019). So currently it is unknown whether ethanol plays a role in the bark beetle system by preferentially fostering the growth of their fungal mutualists.

Ambrosia beetle fungi are not unique in their ability to produce ethanol. In fact, the ability of yeasts and filamentous fungi to produce ethanol is relatively widespread in certain fungal groups (e.g., plant-colonizing fungi like *Fusarium* and *Rhizopus*; the brewer's yeast *Saccharomyces cerevisiae*; saprobic and soil-colonizing fungi like *Aspergillus*) (Schneider and Jeffries, 1989; Singh and Kumar, 1991; Singh et al., 1992; Wainwright et al., 1994; Skory et al., 1997; Ferreira et al., 2014) and has an important role in biotechnologically used fermentation processes. While many of these taxa are able to create their own defended niche by metabolizing, producing and accumulating ethanol in their environment, they are typically found free-living and not in mutualisms with insect hosts (Thomson et al., 2005; Dashko et al., 2014; Zhou et al., 2017). The only other insects known

to be associated with an ethanol-producing fungus (i.e., yeast) are *Drosophila* species, which benefit, like the ambrosia beetles, from the defensive abilities of their symbionts (McKenzie and Parsons, 1972; Becher et al., 2012; Christiaens et al., 2014). Some fungal mutualists of bark beetles like *Endoconidiophora polonica* and *Grosmannia penicillata* (associated with *Ips typographus*), are as well known to produce several different alcohols (Kandasamy et al., 2019), but whether they metabolize, produce and use them to defend themselves like the ambrosia beetle mutualists remains unstudied.

Nutritional fungus mutualisms have evolved independently in at least eleven lineages of wood-boring weevils (Coleoptera: Curculionidae: Scolytinae and Platypodinae) with at least five lineages of ascomycetes (Ophiostomataceae, Nectriaceae, Bionectriaceae, Saccharomycetaceae, Ceratocystidaceae) as well as two lineages of basidiomycetes (Peniophoraceae, Meruliaceae) (Alamouti et al., 2009; Li et al., 2014; Hulcr et al., 2015; Bateman et al., 2017; Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). The ancestral habit of these weevils is phloem and most lineages transitioned to colonize xylem after the origin of the nutritional mutualism (which made it possible to live on the nutrient-poor xylem), possibly because of the high competition within the phloem (Kirkendall et al., 2015; Biedermann and Vega, 2020). Currently, only a few ambrosia beetle fungal mutualists in the Ophiostomataceae and Ceratocystidaceae have been investigated for their affinity to ethanol (Ranger et al., 2018) and it remains unknown how widespread this trait is for the other lineages of ambrosia fungi as well as and in particular for the ancestral fungal symbionts of bark beetles.

Some bark and ambrosia beetles are severe pests, causing high economic damages in forests and plantations worldwide (Kühnholz et al., 2001; Hulcr and Dunn, 2011; La Spina et al., 2013; Ranger et al., 2015; Biedermann et al., 2019). Therefore, research on the physiology of their fungal mutualists is very important to understand the ecology of these beetle-fungus interactions, which may help at some point to develop new management tools that target the fungal mutualists. Here, we examined 10 fungal species from four convergently evolved clades of bark and ambrosia beetle associated fungal symbionts, including five nutritional and two tree-chemistry-detoxifying mutualists and one symbiont with unknown role as well as 2 ubiquitous antagonistic fungi for their ability to grow on ethanol-containing substrate. Two free-living, non-mutualistic filamentous fungi in the same lineages were included to test for phylogenetic effects of ethanol tolerance. Our aims were (i) to test the ethanol tolerance of previously not-tested clades of bark and ambrosia beetle fungal mutualists in the basidiomycete Russulales and the ascomycete Hypocreales and Ophiostomatales (see **Figure 1**) and (ii) to compare it between bark beetle (*sensu stricto*) and ambrosia beetle fungal partners. The latter can help to understand the evolution of ethanol tolerance because ambrosia beetle fungal mutualists are descendants of bark beetle fungal mutualists, which have not been investigated for their tolerance toward ethanol so far. Finally, we aim to test (iii) how widespread the ethanol sensitivity is in antagonists of bark and ambrosia beetles and free-living fungi in the same clades of known beetle mutualists.

MATERIALS AND METHODS

Fungal Strains

In this study, we used common fungal mutualists/symbionts of widespread bark and ambrosia beetles from the three ascomycete orders [Microascales (Ceratocystidaceae), Ophiostomatales (Ophiostomataceae), Hypocreales (Nectriaceae)] and one basidiomycete order [Russulales (Peniophoraceae)]. Additionally, we used two common fungal antagonists of bark and ambrosia beetles in the two ascomycete orders Hypocreales (Cordycipitaceae) and Sordariales (Chaetomiaceae). Finally, we included the nematophagous fungus *Esteya vermicola* in the Ophiostomatales (Ophiostomataceae) as well as the basidiomycete *Entomocorticium sp.* (see Lehenberger et al., 2018) in the Russulales (Peniophoraceae), which served as non-beetle-associated, free-living, phylogenetic controls (for an overview of all strains see **Table 1** and **Supplementary Table S1**). We included all fungal species within a phylogenetic replacement that aimed to (i) visualize the independently evolved phylogenetic lineages of all tested species and to (ii) indicate each classification as well as (iii) our current findings.

A stock collection of all these fungal isolates in glycerol (80%) and glycerol/peptone (80%/1%; Roth, Germany) is constantly maintained at −80°C in our lab in Freiburg, Germany. Origins of the fungal isolates are given in **Supplementary Table 1**. At the beginning of the experiment, we first revived fungi on malt extract agar plates (MEA: 3% malt extract, 0.5% soy peptone, 2% agar, pH = 5.5–6; Sigma-Aldrich, Germany) and stored them at 5°C until they were sub-cultured once more on MEA (25°C, 60% RH) for 5 (fast growers) to 14 (slow growers) days (**Supplementary Table 3**) until the experiment was started for each fungus individually.

DNA Extraction and LSU/βT/ITS Barcoding

We first homogenized fungal samples (pure biomass) by grinding in liquid nitrogen and then proceeded with DNA extraction using the NORGEN Biotek Corp. fungi/yeast genomic DNA isolation kit following the manufacturer's protocol. Sequences of the large subunit (LSU) of the ribosomal RNA gene were primarily used for the identification and phylogenetic placement of isolates (**Figure 1**; **Supplementary Table 1**). For LSU amplification, we used the common primers LROR-F (GTACCCGCTGAACTTAAGC) and LR5-R (ATCCTGAGGGAAACTTCG) (Vilgalys and Hester, 1990; Rehner and Samuels, 1994), which amplify a region of approximately 830 to 880 bp.

Because some samples repeatedly failed to amplify using the LSU primers, we barcoded them using the internal transcribed spacer (ITS) of the ribosomal RNA or the beta-tubulin gene (βT). The ITS region was amplified using primers ITS1-F (TCCGTAGGTGAACCTGCGG) and ITS4-R (TCCTCCGCTTATTGATATGC) (White et al., 1990) and with an annealing temperature of 54.5°C. The ITS region finally contained approximately 580 bp. For amplification of βT, we used the primers T10-F (ACGATAGGTTCACCTCCAGAC)

FIGURE 1 | Phylogenetic placement based on 49 fungal LSU sequences. Black-colored species were examined within this study (*N* = 12), while species in gray act as a fungal outgroup (*N* = 37). The LSU region of the following fungi was sequenced by us and used for the phylogenetic analysis (strain IDs in brackets): *Ophiostoma bicolor* (P22), *Grosmannia penicillata* (2), *Endoconidiophora polonica* (F5), *Beauveria bassiana* (P13), *Entomocorticium* sp. (P8), *Entomocorticium dendroctoni* (P163), and *Fusarium euwallaceae* (P170). Sequences from all other species were obtained from NCBI GenBank. For accession numbers and further information of each individual strain see **Supplementary Tables 1, 2**. Ophiostomatales were divided into their four sub-clades that are currently hypothesized to have evolved independently as nutritional mutualists of ambrosia beetles (Biedermann and Vega, 2020). All examined species were classified into (i) ambrosia beetle fungi, (ii) bark beetle fungi, and (iii) free-living fungi. Triangles denote significant effects on fungal biomass (decrease, increase or not significant; $p < 0.05$) between the 0 and 1% EtOH treatments. Nutritional mutualists of bark and ambrosia beetles are underlined. Due to its abundant sporulation we could not receive comparable data from *Beauveria bassiana* (indicated with *) and excluded it from all further analyses.

and Bt2b-R (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997) and 57.5°C as annealing temperature. Sequence lengths contained around 300 to 400 bp.

For all PCR reactions, we used a similar master-mix for LSU, βT and ITS barcoding [Master-mix for 50 μl: 25 μl 2× phusion high-fidelity PCR master mix with GC buffer (Thermo Scientific™, Germany), 2.5 μl forward primer 1 (10 μM, Eurofins Genomics, Germany) 2.5 μl reverse primer 2 (10 μM, Eurofins Genomics, Germany), 18 μl ddH20, 2 μl template (usually 1:10 diluted)]. For all reactions we applied the following PCR conditions: 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 55.5°C, 54.5°C or 57.5°C (for LSU, ITS, and βT, respectively) for 30 s and 72°C for 20 s, ending with 72°C for 10 min and ending with a storage temperature of 5°C.

DNA-purification was done using the Wizard® SV gel and PCR clean-up system (Promega, Germany) after performing gel electrophoresis. Sanger-sequencing was performed by Eurofins (Eurofins Genomics, Germany). To verify each sequence quality, we used the "SnapGene® Viewer" 3.2 (SnapGene software, from GSL Biotech; available at snapgene.com) and manually corrected automatically deduced sequences, if necessary. Fungal species were finally identified using BLASTn at NCBI (Altschul et al., 1990). All obtained fungal sequences were uploaded to the NCBI GenBank database (the accession numbers are included in **Supplementary Table 1**).

TABLE 1 | Overview of the twelve fungal isolates used in this study and additional information on their classification, beetle host(s), association, phylogenetic order and literature.

Fungal species	Classification	Beetle host(s)	Association	Phylogenetic order	References
Entomocorticium sp.	Free-living	None	None	Russulales	Lehenberger et al., 2018
Entomocorticium dendroctoni	Bark Beetle	*Dendroctonus ponderosae*	Nutritional mutualist	Russulales	Whitney et al., 1987
Endoconidiophora polonica	Bark Beetle	*Ips typographus*	Tree-defenses-detoxifying mutualist	Microascales	De Beer et al., 2014
Ophiostoma bicolor	Bark Beetle	*Ips* spp.	Symbiont with unknown role	Ophiostomatales (Clade 1)	Davidson, 1955
Grosmannia penicillata	Bark Beetle	*Ips typographus*	Tree-defenses-detoxifying mutualist	Ophiostomatales (Clade 3)	Goidanich, 1936
Fusarium euwallaceae	Ambrosia Beetle	*Euwallacea fornicatus*	Nutritional mutualist	Hypocreales	Freeman et al., 2013
Ambrosiella hartigii	Ambrosia Beetle	*Anisandrus dispar*	Nutritional mutualist	Microascales	Batra, 1967
Raffaelea canadensis	Ambrosia Beetle	*Xyleborinus saxesenii*	Nutritional mutualist	Ophiostomatales (Clade 2)	Batra, 1967
Raffaelea sulphurea	Ambrosia Beetle	*Xyleborinus saxesenii*	Nutritional mutualist	Ophiostomatales (Clade 4)	Harrington et al., 2010
Chaetomium globosum	Pathogen	*Various*	Resource competitor	Sordariales	Fries, 1829
Beauveria bassiana	Pathogen	*Various*	Entomo-pathogen	Hypocreales	Vuillemin, 1912
Esteya vermicola	Free-living	None	None	Ophiostomatales (Clade 4)	Liou et al., 1999

Phylogenetic Analysis

Sequences of the LSU for seven of our fungal sequences were used for maximum likelihood analyses and construction of a phylogenetic tree (**Supplementary Table 1**). NCBI GenBank entries were used for the remaining five sequences of studied fungi as well as for 37 fungal outgroup sequences (**Supplementary Table 2**). Individual sequences were prepared using GeneDoc 2.7 (Nicholas, 1997) or MEGA 7.0 (Kumar et al., 2016) and were additionally compared with the received output from Clustal Omega (Madeira et al., 2019). The phylogenetic analyses were done with the IQ-Tree-Web-Server (Nguyen et al., 2015; Trifinopoulos et al., 2016) using the ultrafast bootstrap analysis (Minh et al., 2013). Based on the web server FindModel (Posada and Crandall, 1998, available at[1]), we chose the generalized time reversible (GTR) model plus gamma (+ G) with rate heterogeneity (rate categories – 4) and combined it with 8.000 bootstrap alignments as our substitution model. FigTree[2] was used for visualization of the phylogenetic tree.

The phylogenetic relationship of tested fungi is already known (see Rollins et al., 2001; Alamouti et al., 2009; Dreaden et al., 2014; Vanderpool et al., 2018), therefore we did not include it within the results. The aim of the phylogenetic reconstruction was to give an overview over the different examined clades with our choice of fungi as well as to visually summarize our findings in an appealing way (see **Figure 1**).

Ethanol-Based Culturing

We examined the influence of five ethanol (EtOH) concentrations in MEA culturing media (0, 1, 2, 3, 5 vol/vol) on biomass, area and density of 12 fungal strains. The methods closely followed those reported in Ranger et al. (2018). Ethanol (99.8%, Sigma-Aldrich, Germany) was added to the MEA media at around 55°C (just before it solidified) to reduce its evaporation. Immediately after the media had cooled down in the petri dishes, we added a sterile cellophane membrane (6 × 6 cm, NeoLab, Germany) and a plug of the pre-cultured fungal mycelium (Ø 3 mm, using a cork borer) to the center of each plate. We used eight replicates per fungus for each of the five EtOH treatments, for a total of 40 plates per fungus, which were sealed with parafilm and subsequently incubated in dark at 25°C and 60% RH. Images of the colonies were taken every second day (Sony alpha 5000, 12 × in macro mode) through the closed lid of the petri dish. Only at the last day of the experiment the lid was removed before taking the picture (see **Figure 2** and **Supplementary Figure 1**). All images were analyzed for surface area using ImageJ software (version 1.52a). The experiment was terminated for each fungal species separately and as soon as one replicate (independent of the EtOH treatment) reached an edge of the cellophane membrane. For individual incubation times see **Supplementary Table 3**. If single replicates were contaminated, we excluded them from our analyses (**Supplementary Table 3**). After termination of the experiment and taking of the picture, the entire fungal biomass was collected from each cellophane membrane, dried individually at 50°C for up to 14 days (dry biomass) in a drying oven and was then weighed. To calculate the density (mg/mm^2) of fungal mycelia, the determined dry weight (mg) was divided by measured surface area (mm^2).

Effects of EtOH concentration on dry biomass, surface area and density for each fungal species were visualized with ggplot in R (version 1.2.5033) using the package ggplot2 (Wickham, 2016). One-way-ANOVAs (log10 transformed data; Biomass in dependence of ethanol concentration) were conducted to detect differences in the biomass between the 0 and 1%, and the 0 and 2% EtOH treatments for each fungus using the package rcompanion (CRAN.R-project.org/package = rcompanion). We added the received ANOVA output for each examined fungus to the Anova Output.

RESULTS

Fungal Biomass in Relation to the Amount of EtOH in the Media

Ethanol enriched substrate had a strong effect on the majority of examined fungal species. Three different reactions of tested fungi toward EtOH could be distinguished: (I) Fungal biomass

[1] hiv.lanl.gov/content/sequence/findmodel/findmodel.html

[2] http://tree.bio.ed.ac.uk/software/figtree

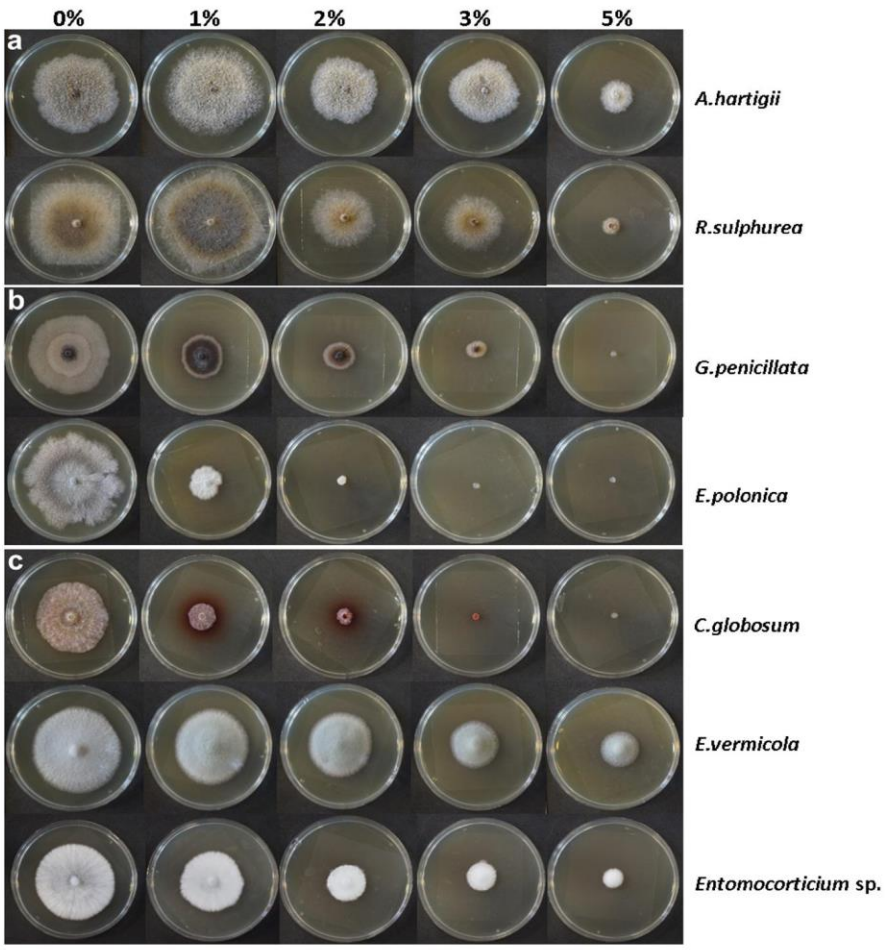

FIGURE 2 | . Representative images of seven studied fungi growing on MEA treated with five investigated ethanol concentrations (0, 1, 2, 3, and 5% vol/vol). **(a)**: ambrosia beetle fungi = *Ambrosiella hartigii*, *Raffaelea sulphurea*; **(b)**: bark beetle fungi = *Grosmannia penicillata*, *Endoconidiophora polonica*; **(c)**: pathogens/free-living = *Chaetomium globosum*, *Esteya vermicola*, *Entomocorticium* sp. For incubation times and further information of each individual strain see **Supplementary Table 3**. The five remaining fungal species are displayed in **Supplemantary Figure 1**.

was positively affected (**Figure 3A**) (II) not affected (**Figure 3A**) or (III) negatively affected (**Figure 3B**) by medium containing 1% ethanol in comparison to the absence of ethanol. Fungal species were only associated with pattern I or III, if a significant difference between the 0 and 1% EtOH treatment could be detected. In case we did not detect any significances, fungi were generally associated with pattern II, even though some species showed a slight non-significant increase or decrease of biomass.

Within the Microascales, we observed significantly more fungal biomass at 1% EtOH (pattern I) for the nutritional mutualist of the ambrosia beetle *Anisandrus dispar*, *Ambrosiella hartigii* (p = 0.0008, **Figure 2a**) while the detoxifying mutualist of the bark beetle *Ips typographus*, *Endoconidiophora polonica* (p = 2.23e-10, **Figure 2b**) produced significantly fewer biomass and thus showed pattern III. In the Opiostomatales, the non-beetle-associated, endophyte *Esteya vermicola* (p = 0.008, **Figure 2c**) clearly showed pattern I while there was no significant increase in biomass but a general tolerance toward ethanol (pattern II) for the two nutritional mutualists of the ambrosia beetle *Xyleborinus saxesenii*, *Raffaelea sulphurea* (p = 0.65, **Figure 2a**) and *R. canadensis* (p = 0.35, **Supplementary Figure 1B**), (mean biomass for both was higher at 1% EtOH, but not statistically significant). The tree-defenses-detoxifying mutualist of *I. typographus*, *Grosmannia penicillata* (p = 0.154, **Figure 2b**) showed a tolerance toward the 1% EtOH treatment, even though mean biomass decreased with increasing EtOH concentrations (no significance at 1% EtOH (pattern II), but rather similar to pattern III). Another symbiont with unknown role in *I. typographus*, *Ophiostoma bicolor* (p = 4.01e-11, **Supplementary Figure 1C**), was highly negatively affected by EtOH (pattern III). The only Sordariales, the antagonistic bark and ambrosia beetle symbiont *Chaetomium globosum* (p = 1.83e-06, **Figure 2c**) produced significantly fewer biomass (pattern III). Within the Russulales, the supposedly free-living, endophytic *Entomocorticium* sp. (isolated from *Trypodendron*

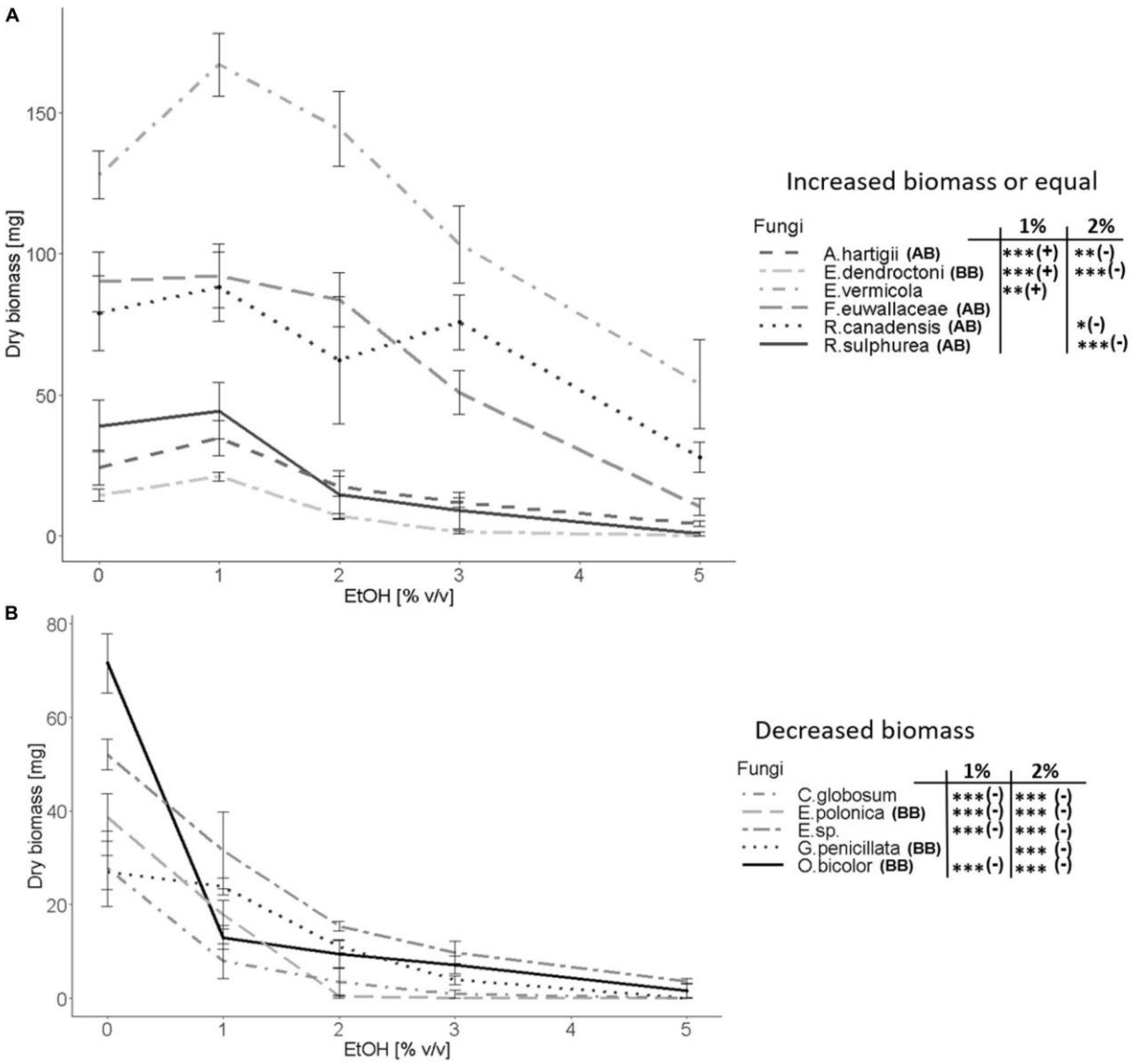

FIGURE 3 | Fungal biomass in mg (dry-weight) as a function of the EtOH (% v/v) treatment (0, 1, 2, 3, and 5% vol/vol). Fungi were classified into **(A)** positively affected (*Ambrosiella hartigii*, *Entomocorticium dendroctoni*, *Esteya vermicola*) and not affected (*Raffaelea canadensis*, *R. sulphurea Fusarium euwallaceae*) by the 1% EtOH treatment or **(B)** negatively affected (*Chaetomium globosum*, *Endoconidiophora polonica*, *Entomocorticium sp.* (E.sp.), *Grosmannia penicillata*, *Ophiostoma bicolor*) by the 1% EtOH treatment. With one exception (*E. vermicola*) all species in the first group are nutritional mutualists of bark and ambrosia beetles, whereas all species in the second group are either plant-defenses-detoxifying mutualists of bark beetles, free-living or antagonistic species. (AB) denotes ambrosia beetle associates and (BB) bark beetle associates. Means and standard deviations are given for each EtOH treatment. Significant differences between the 0% and 1% and 2% EtOH treatments are given: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Additionally, we added (+) and (–) to indicate a significant increase, respectively, decrease of fungal biomass. $N = 8$ replicates per fungal species.

lineatum) shows pattern III (p = 1.73e-06, **Figure 2c**), while the nutritional mutualist of the bark beetle *Dendroctonus ponderosae, Entomocorticium dendroctoni* shows pattern I (p = 0.00003, **Supplementary Figure 1C**). In the Hypocreales, the only fungal species which was tolerating EtOH even up to 2% (pattern II) is the nutritional mutualist of the ambrosia beetle *Euwallacea fornicatus*, *Fusarium euwallaceae* (p = 0.82, **Supplementary Figure 1B**). Within the same group, the generally entomopathogenic fungus *Beauveria bassiana* (**Supplementary Figure 1A**) showed an enormous standard deviation due to its abundant sporulation, which made it impossible to compare the biomass of this species at different ethanol concentrations as spores easily spread all over the plate. Its growth patterns should be treated with care and are thus only presented in **Supplementary Table 3** and **Supplementary Figure 1A**. All examined fungi produced less biomass above 2 to 3% EtOH

compared to the 0% EtOH treatment. This effect was particularly present in the bark beetle symbionts (*E. polonica*, *O. bicolor*, *G. penicillata*, *E. dendroctoni*), the antagonist *C. globosum* and the endophyte *Entomocorticium* sp., while the remaining ambrosia beetle mutualists were less strongly affected. The strongest effect could be observed at the 5% EtOH media, on which only ambrosia beetle mutualists as well as the free-living endophyte *E. vermicola* produced substantial amounts of biomass (see **Figure 2**, **Supplementary Figure 1** and **Supplementary Table 3**). Results from fungal surface area as well as the calculated density in relation to the amount of EtOH in the media can be found in the **Supplementary Results**.

DISCUSSION

Our findings showed that the examined obligate and nutritional ambrosia beetle mutualists (*A. hartigii*, *R. canadensis*, *R. sulphurea*) as well as the obligate and nutritional bark beetle mutualist (*E. dendroctoni*) benefit or at least are not harmed (*F. euwallaceae*) by the presence of low (1–2%) concentrations of ethanol. Most fascinating, this tolerance of ethanol by mutualistic fungi is apparently not restricted to a specific lineage of ambrosia beetle fungi (**Figure 1**), but must have evolved convergently in different orders of fungi, such as the ascomycete Microascales, Ophiostomatales and Hypocreales as well as the basidiomycete Russulales. Interestingly, the majority of non-nutritional, plant-defenses-detoxifying bark beetle fungi (*E. polonica*, *G. penicillata*, *O. bicolor*) and the fungal antagonist of bark and ambrosia beetles *C. globosum* strongly decrease in biomass in the presence of ethanol. Thus, we confirm and further expand the findings of Ranger et al. (2018) (that were based on just a handful of fungi) to some more lineages of independently evolved bark and ambrosia beetle symbionts with beneficial to antagonistic roles. Our findings show that non-nutritional mutualists of bark beetles (which have never been investigated for their ethanol tolerance) are sensitive to even small amounts of ethanol. This is fascinating because at least in the Ophiostomatales and Microascales these fungi are ancestral to ambrosia beetle mutualists, suggesting that ethanol tolerance convergently evolved repeatedly along with the xylem-boring and fungus-farming habit of ambrosia beetles. Interestingly, all symbionts of bark and ambrosia beetles can tolerate or benefit by the presence of 1–2% ethanol if they are nutritionally beneficial to their obligately dependent beetle host. This is particularly interesting because we have pairs of ethanol benefiting/tolerant vs. sensitive fungal species in almost every fungal order that we tested. It indicates that ethanol tolerance is a derived trait in bark and ambrosia beetle nutritional mutualists that are transmitted in mycetangia and actively farmed by their hosts. Furthermore, the ethanol preference means that these fungi (as well as their hosts) are adapted to colonize stressed or recently dead trees (Moeck, 1970; Miller and Rabaglia, 2009; Ranger et al., 2011, 2013, 2015, 2018; Reding et al., 2011).

While the group of bark and ambrosia beetles are best known by their tree-killing species, the majority of species actually colonizes highly stressed or recently dead host trees (Batra, 1963; Beaver et al., 1989; Kühnholz et al., 2001; Hulcr and Dunn, 2011; Kirkendall et al., 2015; Ranger et al., 2015). On the tree, they differ in the localization of their tunnels – xylem for ambrosia and phloem for bark beetles – and their association with fungi that are typically nutritionally important for ambrosia beetles and detoxify (or induce) tree defenses for bark beetles (but see exceptions in some *Dendroctonus* and *Ips* spp.) (Batra, 1963, 1967; Francke-Grosmann, 1963, 1966; Whitney et al., 1987; Kirkendall et al., 2015; Hulcr and Stelinski, 2017; Vissa and Hofstetter, 2017; Kandasamy et al., 2019). As we discussed above, ambrosia beetles are generally associated with ethanol tolerant or benefiting fungi, whereas bark beetle fungi are typically sensitive to ethanol. This is also reflected by the attraction to ethanol during tree-host finding only of ambrosia beetles, but not of bark beetles. Studies show that ethanol can be found in both xylem and phloem tissue (Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1991; Kelsey et al., 2014) and ethanol is even enriched in the plant tissues during bark beetle attacks (Kelsey et al., 2014). However, it is unclear if phloem of dying trees is generally lower in ethanol (e.g., due to evaporation) or if bark and ambrosia beetles differ in host substrate preferences. Also, it is possible that both bark and ambrosia beetles have to be exposed to ethanol in their breeding substrate, but that bark beetle symbionts (e.g., bacteria) are capable of detoxifying the phloem. A variety of yeasts, bacteria and filamentous fungi have been isolated from bark beetles (Six, 2013) and many of them have detoxifying capabilities (Dowd, 1989, 1992; Skrodenyte-Arbaciauskiene et al., 2006; Hammerbacher et al., 2013; Um et al., 2013; Ramadhar et al., 2014; Wadke et al., 2016; Zhao et al., 2019).

All fungi showing a tolerance (*R. sulphurea*, *R. canadensis*, *F. euwallacea*, *G. penicillata*) or even an increase in biomass at 1% ethanol (*E. vermicola*, *A. hartigii*, *E. dendroctoni*, **Figure 3A**) might indicate an elevated activity of alcohol dehydrogenases (ADHs; referring to the ADH experiment in Ranger et al., 2018). These enzymes allow organisms to detoxify ethanol and even consume it as a carbon source, which can lead to an increase in biomass in the presence of EtOH, as we found in some species (significant increase: *E. vermicola*, *A. hartigii*, *E. dendroctoni*; not significant, but higher mean biomass: *R. canadensis*, *R. sulphurea*, F. euwallaceae). The presence and high activity of ADHs has already been supported by Ranger et al. (2018) for the ambrosia beetle fungal mutualists *Ambrosiella grosmanniae* and *Raffaelea canadensis*, while ADHs were only poorly present in *Ambrosiella roeperi* and not detectable within the yeast-like fungus *Ascoidea* sp. and the fungal antagonist *Aspergillus* sp. Ranger et al. (2018) found a significant increase in biomass at 1% ethanol for *R. canadensis* that we did not find.

Our findings regarding the sensitivity of plant-defenses-detoxifying mutualists of bark beetles toward ethanol (pattern III) in contrast to the insensitivity of nutritional mutualists of bark and ambrosia beetles (pattern I and II) should be interpreted with care, however. Here, we tested only a small fraction of fungi, with only one nutritional mutualist of a bark beetle and no plant-defenses-detoxifying mutualists of ambrosia beetles. Therefore, it is currently unclear whether the ethanol sensitivity that is certainly more common in bark beetle symbionts is

related to the phloem habitat or the beneficial physiology of the fungi to the beetles.

In the Hypocreales, the fourth lineage of ambrosia beetle fungal mutualists, *F. euwallaceae*, was neither facilitated nor harmed by EtOH up to 2% relative to the control (**Figure 3A**). This indicates that this fungus can tolerate EtOH, which is already known for the genus *Fusarium*; for example, *F. oxysporum* is known to produce ethanol (Ueng and Gong, 1982; Christakopoulos et al., 1989). This might indicate that the insensitivity toward ethanol had been present at the origin of the ambrosia beetle-fungus mutualism in the Hypocreales and did not evolve anew as hypothesized for the other lineages (see above). The endophytic ascomycete *E. vermicola* (Ophiostomatales) (**Figure 3A**) can also metabolize ethanol, similar to ambrosia beetle fungal mutualists. This is especially interesting as this fungus is not associated with any beetle (Liou et al., 1999), but is phylogenetically closely related to the ambrosia beetle fungal mutualist *R. sulphurea* (**Figure 1**). This could mean that ethanol tolerance is present in some free-living fungi, or even that *E. vermicola* has been previously associated with beetles. Close examination of the phylogeny of the relatives of *E. vermicola* and *R. sulphurea* and their EtOH sensitivities have to be conducted to answer this question.

Quite unique for filamentous fungal species is the high tolerance (reflected by the ability to produce some biomass) of some ambrosia beetle mutualists (*A. hartigii*, *R. canadensis*, and *F. euwallaceae*) as well as some free-living fungi (*E. vermicola* and *Entomocorticium* sp.) toward even the highest tested EtOH concentration (5%). (**Figures 2a,c** and **Supplementary Figure 1B**). This tolerance is close to the naturally occurring, wild-type strains of yeasts (up to 6% ethanol by certain species in the genus *Saccharomyces*, *Candida*, *Fabospora*, *Kluyveromyces*, *Kloeckera*) and of specific filamentous fungi (3–7% by certain species in the genus *Rhizopus* and *Fusarium*) used for producing ethanol and above the typical limits of alcohol tolerance in other fungal species (Gao and Fleet, 1988; Singh and Kumar, 1991; Singh et al., 1992; Skory et al., 1997; Banat et al., 1998; Pina et al., 2004; Benjaphokee et al., 2012; Ferreira et al., 2014; Lam et al., 2014; Paschos et al., 2015; Ruchala et al., 2020). In biotechnology, ethanol tolerance and production of fungi can be increased by culture dependent methods (e.g., pH, temperature, medium, carbon-source), genetic modifications and artificial selection (e.g., Gao and Fleet, 1988; Skory et al., 1997; Pina et al., 2004; Benjaphokee et al., 2012; Lam et al., 2014; Ruchala et al., 2020). The extraordinary tolerance of some of our tested fungi in combination with the known ethanol production of ambrosia beetle fungi makes them quite interesting for biotechnological purposes (i.e., second-generation biofuels made from plant biomass). Moreover, as we show here, their tolerance toward ethanol evolved apparently several times independently in unrelated fungal lineages, so it might be fruitful for biotechnological purposes to investigate whether the tolerance is always enabled by the same physiological mechanisms.

Here, we show that not only specific ambrosia beetles, but many ambrosia beetle species and even a *Dendroctonus* bark beetle rely on fungal partners that detoxify and metabolize ethanol. This competitive ability of their mutualistic partners may allow the beetles to indirectly select their partners by biological screening through the ethanol-containing substrate they choose for boring their galleries (Scheuring and Yu, 2012; Ranger et al., 2018). By contrast, most non-nutritional bark beetle fungal mutualists lack the ability to tolerate ethanol. Further studies are necessary to test whether ethanol is generally absent in most of the phloem colonized by bark beetles or is degraded by other symbionts in the microbiome of the beetles.

DATA AVAILABILITY STATEMENT

All obtained fungal sequences were uploaded to the NCBI GenBank database (the accession numbers are included in **Supplementary Table 1**).

AUTHOR CONTRIBUTIONS

ML and PB designed the study and wrote the manuscript. ML isolated and sequenced all examined fungi, analyzed data, did statistics, plotted results, and constructed the phylogenetic tree. MB and ML did the ethanol-based culturing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.590111/full#supplementary-material

Supplementary Figure 1 | Representative images of five studied fungi growing on MEA treated with five investigated ethanol concentrations (0, 1, 2, 3, and 5% vol/vol). **(A)**: entomopathogens = *Beauveria bassiana*; **(B)**: ambrosia beetle

fungi = *Raffaelea canadensis*, *Fusarium euwallaceae*; **(C)**: bark beetle fungi = *Entomocorticium dendroctoni*, *Ophiostoma bicolor*. For incubation times and further information on each individual strain see **Supplementary Table 3**. The seven other fungal species are displayed in **Figure 1**.

Supplementary Figure 2 | (A) = Surface area in mm^2 and **(B)** = density in mg/mm^2 based on EtOH treatment (0, 1, 2, 3, 5 vol/vol) of fungi positively affected (*Ambrosiella hartigii*, *Entomocorticium dendroctoni*, *Esteya vermicola*) and not affected (*Raffaelea canadensis*, *R. sulphurea, Fusarium euwallaceae*) in dry weight (see **Figure 3**) by the 1% compared to the 0% EtOH treatment. Means and standard deviations are given. *N* = 8 replicates per fungal species.

Supplementary Figure 3 | (A) = Surface area in mm^2 and **(B)** = density in mg/mm^2 based on the EtOH treatments (0, 1, 2, 3, 5%) of fungi negatively affected (*Chaetomium globosum*, *Endoconidiophora polonica*, *Entomocorticium* sp. (E.sp.), *Grosmannia penicillata*, *Ophiostoma bicolor*) in dry weight (see **Figure 3**) by the 1% compared to the 0% EtOH treatment. Means and standard deviations are given. *N* = 8 replicates per fungal species.

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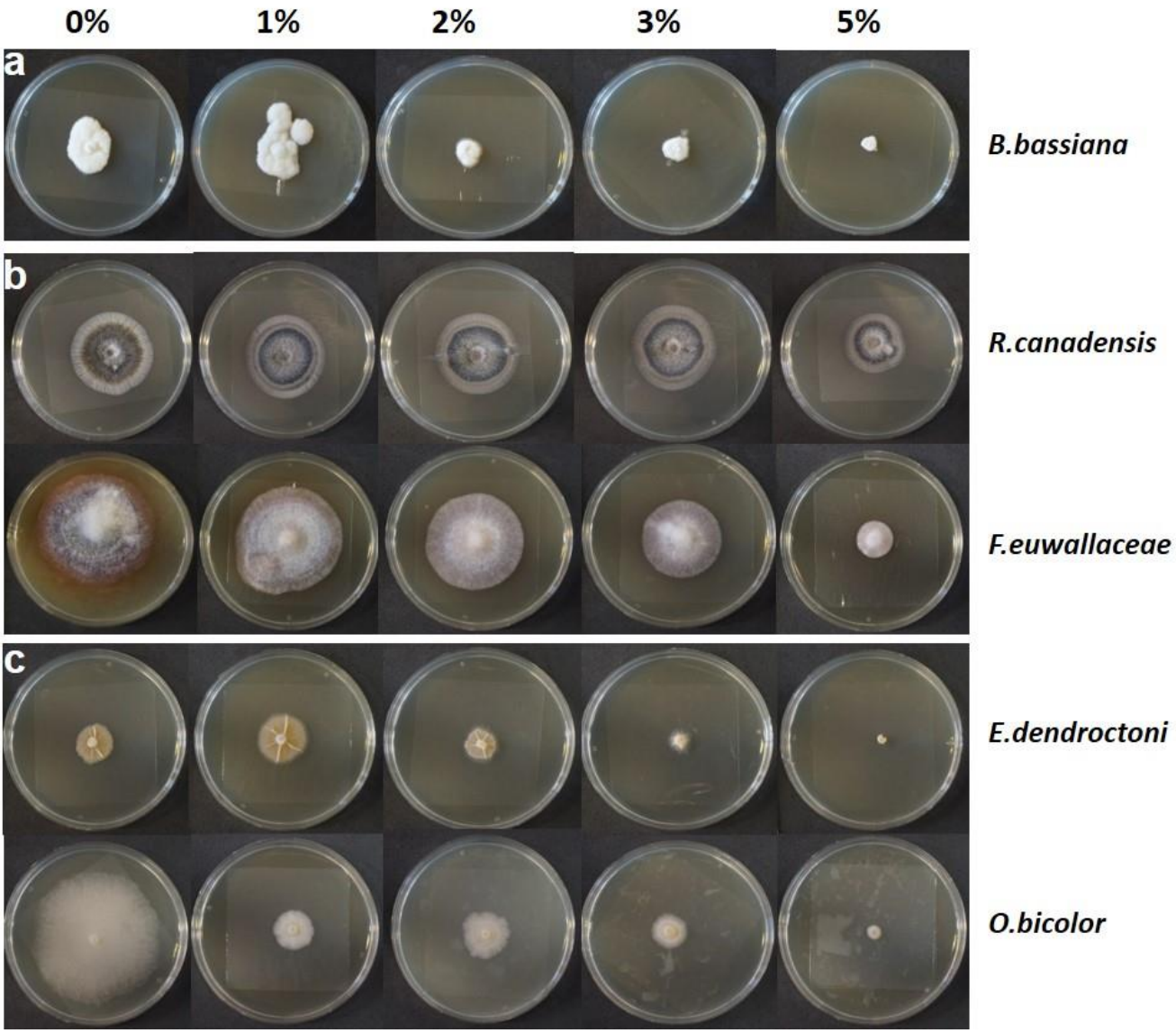
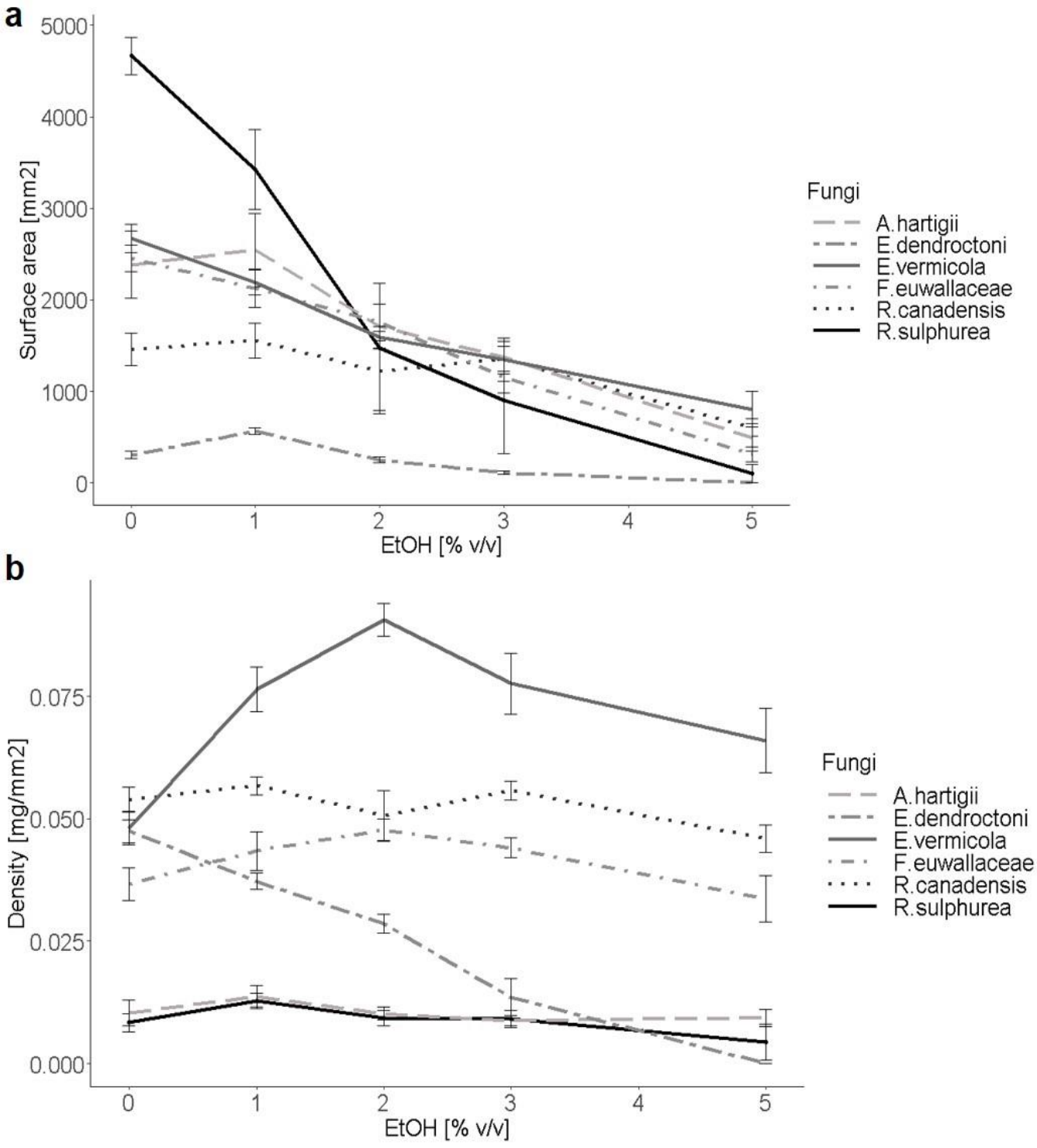
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

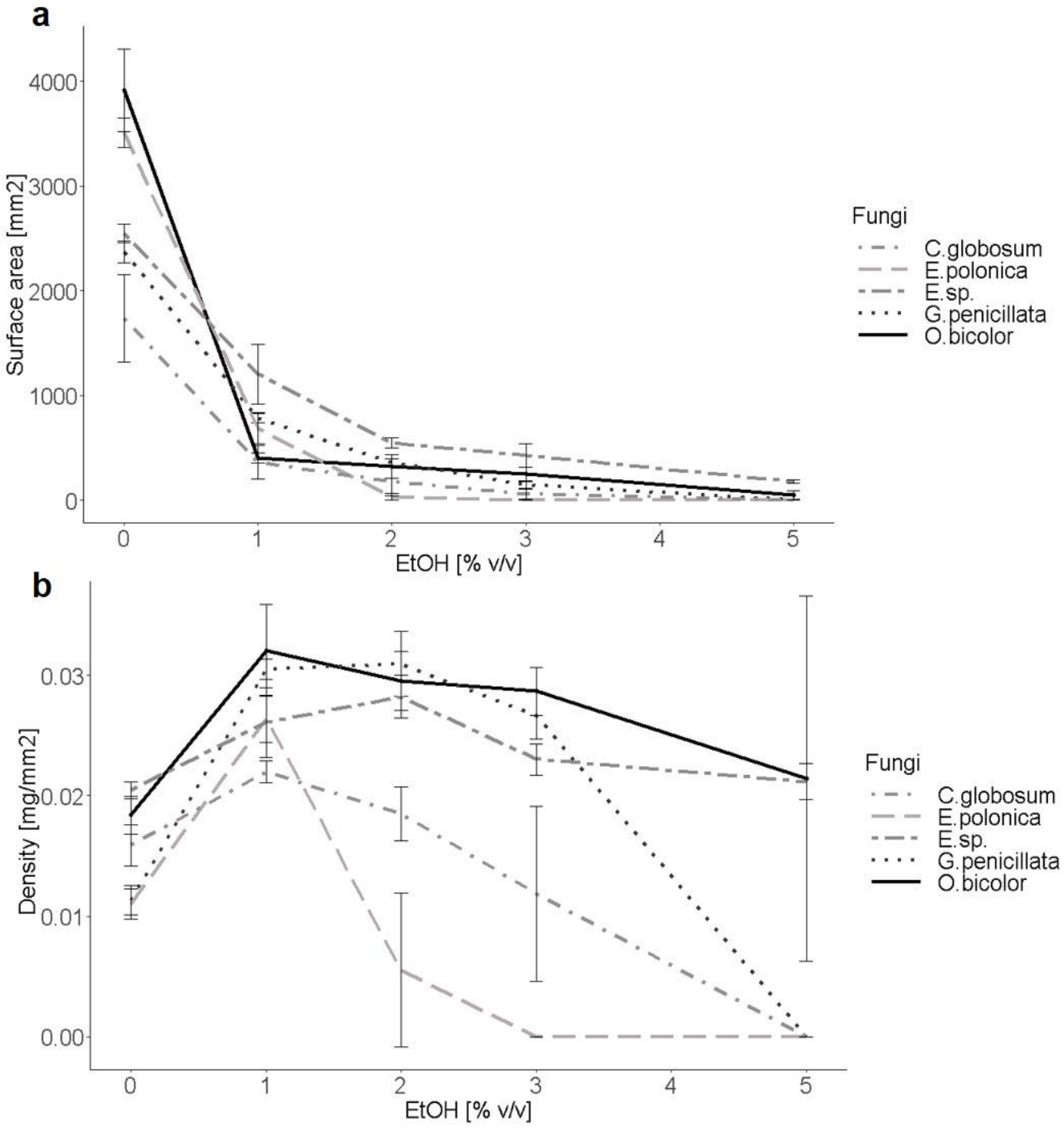
Annex – Supplementary Figures



Suppl. Figure 1: Representative images of five studied fungi growing on MEA treated with five investigated ethanol concentrations (0%, 1%, 2%, 3% and 5% vol/vol). (a): entomopathogen = *Beauveria bassiana*; (b): ambrosia beetle fungi = *Raffaelea canadensis*, *Fusarium euwallaceae*; (c): bark beetle fungi = *Entomocorticium dendroctoni*, *Ophiostoma bicolor*. For incubation times and further information on each individual strain see Table S3. The seven other fungal species are displayed in Fig. 1.



Suppl. Figure 2: (a) = Surface area in mm2 and (b) = density in mg/mm2 based on EtOH treatment (0%, 1%, 2%, 3%, 5% vol/vol) of fungi positively affected (*Ambrosiella hartigii*, *Entomocorticium dendroctoni*, *Esteya vermicola*) and not affected (*Raffaelea canadensis*, *R. sulphurea*, *Fusarium euwallaceae*) in dry weight (see Fig. 3) by the 1% compared to the 0% EtOH treatment. Means and standard deviations are given. N = 8 replicates per fungal species.



Suppl. Figure 3: (a) = Surface area in mm2 and (b) = density in mg/mm2 based on the EtOH treatments (0%, 1%, 2%, 3%, 5%) of fungi negatively affected (*Chaetomium globosum*, *Endoconidiophora polonica*, *Entomocorticium* sp. (E.sp.), *Grosmannia penicillata*, *Ophiostoma bicolor*) in dry weight (see Fig. 3) by the 1% compared to the 0% EtOH treatment. Means and standard deviations are given. N = 8 replicates per fungal species.

Annex – Supplementary Tables

Table S1: Overview of the origin of fungal isolates used in this study.

Species	ID	Year of isolation	Locality	Beetle host	Isolated by	Sequenced marker
Entomocorticium sp.	P8	2016	Neuschönau, Bavaria, Germany	*Trypodendron lineatum*	Lehenberger M; Lehenberger et al. 2018	LSU
Entomocorticium dendroctoni	P163	2018	Arizona, USA	*Dendroctonus brevicomis*	Lehenberger M; following protocol in Lehenberger et al. 2019	LSU
Endoconidiophora polonica	F5	2013	South Karelia, Finland	*Ips typographus*	Unknown	LSU
Ophiostoma bicolor	P22	2004	Akershus, Norway	*Ips typographus*	Unknown	LSU
Grosmannia penicillata	2	2006	Kronoberg county, Sweden	*Ips typographus*	Unknown	LSU
Fusarium euwallaceae	P170	2018	Israel	*Euwallacea fornicatus*	Mendel Z	LSU
Ambrosiella hartigii	P137	2019	Würzburg, Bavaria, Germany	*Anisandrus dispar*	Lehenberger M; following protocol in Lehenberger et al. 2019	ITS
Raffaelea canadensis	P23	2019	Würzburg, Bavaria, Germany	*Xyleborinus saxesenii*	Lehenberger M; following protocol in Lehenberger et al. 2019	Beta-tubulin
Raffaelea sulphurea	P159	2019	Würzburg, Bavaria, Germany	*Xyleborinus saxesenii*	Lehenberger M; following protocol in Lehenberger et al. 2019	Beta-tubulin
Chaetomium globosum	P7	2018	Würzburg, Bavaria, Germany	Gallery of *Xyleborinus saxesenii*	Lehenberger M; following protocol in Lehenberger et al. 2019	ITS
Beauveria bassiana	P13	2019	Würzburg, Bavaria, Germany	*Ips typographus*	Lehenberger M; following protocol in Lehenberger et al. 2019	LSU
Esteya vermicola	CBS156.82	1999	Japan	*Pinus* – dried plant	Liou JY, Shih JY & Tzean	ITS

Table S2: Overview of fungal species used for phylogenetic analysis with the specimen identification number (ID) and the corresponding GenBank accessions for 28s/LSU.

Annotation	ID	Accession number	Beetle host	Reference
Heterobasidion annosum	CBS 859.87	**MH873808**	None	Vu et al. 2019
Peniophora rufa	B1014	**MN475831.1**	None	Harrington et al. 2019 Unpublished
P.crassitunicata	CBS 663.91	**NG_064161**	None	Vu et al. 2019
Entomocorticium sp.	P8	**MT159435**	Unknown	Lehenberger et al. 2020, unpublished
E.dendroctoni	P163	**MT159436**	*Dendroctonus brevicornis*	Lehenberger et al. 2020, unpublished
Penicillium commune	CBS 341.59	**MH869425.1**	None	Vu et al. 2019
Raffaelea fusca	CBS129011	**MH878043.1**	*Xyleborus glabratus*	Vu et al. 2019
R.canadensis	CBS 326.70	**MH871450.1**	*Xyleborinus saxesenii*	Vu et al. 2019
R.quercivora	RA2245	**LC069313.1**	*Platypus quercivorus*	Torii et al. 2016
R.montetyi	PC06.001	**JF909540.1**	*Platypus cylindrus*	Inacio et al. 2011 Unpublished
R.sulphurea	C593	**EU177463.1**	*Xyleborinus saxesenii*	Harrington et al. 2010
R.amase	CBS116694	**EU984295.1**	*Amasa concitatus*	Alamouti et al. 2009
R.lauricola	CBS 121567	**MH877762.1**	*Xyleborus glabratus*	Vu et al. 2019
R.sulcati	CBS 806.70	**NG_064084.1**	*Gnathotrichus sulcatus*	Vu et al. 2019
R.brunnea	MAR50.a	**MN012913.1**	*Monarthrum* spp.	Restrepo et al. 2019 Unpublished
R.rapaneae	CMW40359	**KT182935**	*Lanurgus* sp.	Musvuugwa et al. 2015
R.ambrosiae	C2225	**EU177453**	*Platypus* spp.	Harrington et al. 2010
R.santoroi	CBS 399.67	**NG_064067**	*Platypus* spp.	Vu et al. 2019
Raffaelea quercus-mongolicae	RQM04	**KF513155**	*Platypus koryoensis*	Park. 2013 Unpublished
Esteya vermicola	CBS 115803	**EU668903.1**	None	Wang et al. 2008
Grosmannia clavigera	M002	**GU370280.1**	*Dendroctonus ponderosae*	Roe et al. 2010
G.aurea	CMW667	**DQ294389.1**	*Dendroctonus* spp.	Zipfel et al. 2006
G.francke-grosmanniae	CMW2975	**DQ294395.1**	Unknown	Zipfel et al. 2006
G.penicillata	2	**MT159434**	*Ips* spp.	Lehenberger et al. 2020, unpublished
Leptographium castellanum	CBS 128697	**MH876514.1**	Unknown	Vu et al. 2019
L.abietinum	CF19	**MH084777.1**	*Dendroctonus*	Davis et al.

			rufipennis	2019
L.procerum	CBS 128844	**MH876524.1**	*Dendroctonus* spp.	Vu et al. 2019
L.lundbergii	CBS 128843	**MH878023**	*Ips* spp.	Vu et al. 2019
Ophiostoma dryocoetis	CBS 376.66	**MH870468.1**	*Dryocoetes confusus*	Vu et al. 2019
O.piceae	CBS 180.69	**MH877705.1**	Unknown	Vu et al. 2019
O.quercus	CBS 366.93	**MH874074.1**	*Platypus* spp.	Vu et al. 2019
O.piliferum	CBS 138.33	**MH866835.1**	Unknown	Vu et al. 2019
O.bicolor	P22	**MT159431**	*Ips* spp.	Lehenberger et al. 2020, unpublished
O.montium	CMW13221	**DQ294379.1**	*Dendroctonus ponderosae*	Zipfel et al. 2006
O.ips	9	**KY946730.1**	*Ips pini*	Pastirčáková et al. 2018
Sporothrix schenckii	CBS 359.36	**KX590890.1**	Unknown	De Beer et al. 2016
S.stenoceras	CBS 798.73	**MH872535.1**	Unknown	Vu et al. 2019
Neurospora crassa	ICMP 6360	**AY681158.1**	None	Cai et al. 2006
Chaetomium globosum	CBS 128472	**MH876396.1**	None	Vu et al. 2019
Ambrosiella beaveri	C2749	**KF646765.2**	*Xylosandrus mutilatus*	Harrington et al. 2014
A.grosmanniae	D16	**MG050696.1**	*Xylosandrus germanus*	Van de Peppel et al. 2018
A.hartigii	D17	**MG050697.1**	*Anisandrus dispar*	Van de Peppel et al. 2018
A. roeperi	14766	**MG954244.1**	*Xyleborus crassisculus*	Huang et al. 2018 Unpublished
Endoconidiophora polonica	F5	**MT159432**	*Ips* spp.	Lehenberger et al. 2020, unpublished
E. pinicola	CMW29499	**KM495364.1**	Unknown	De Beer. 2014 Unpublished
Phialophoropsis ferruginea	CBS 460.82	**KM495316**	*Trypodendron lineatum*	De Beer. 2014 Unpublished
Baeuveria bassiana	P13	**MT159433**	None	Lehenberger et al. 2020, unpublished
Fusarium solani	CBS490.63	**AY097316.1**	None	Summerbell and Schroers 2002
F. euwallaceae	P170	**MT159437**	*Euwallacea fornicatus*	Lehenberger et al. 2020, unpublished

Table S3: Overview of the ethanol assays and the results for dry-biomass, covered area and mycelial density for each fungal isolate.
Statistical differences (p) are given only for the dry-biomass and relative to 0% ethanol: ns - p > 0.05, * - p < 0.05, ** - p < 0.001, *** - p < 0.0001; (+) significant increase, (-) significant decrease.

Fungal species	Pre-culture-time [d]	Incubation-time [d]	EtOH-treatment [%]	Replicates [N]	Dry-biomass Mean [mg]	Dry-biomass SD [mg]	Dry-biomass p (0%)	Area mean [mm^2]	Area SD [mm^2]	Density mean [mg/mm^2]	Density SD [mg/mm^2]
Entomocorticium sp.	8	7	0	8	52.1	3.2		2547.5	84.87	0.0204	0.0007
			1	8	31.6	8.2	***(-)	1202.6	285.22	0.0261	0.0029
			2	8	15.4	1	***(-)	548.4	46.88	0.0282	0.0018
			3	8	9.8	2.4	***(-)	426.8	110.1	0.023	0.0013
			5	8	3.7	0.5	***(-)	176.1	14.3	0.0212	0.0015
E. dendroctoni	14	14	0	8	14.5	2.1		305.2	43.228	0.0474	0.0023
			1	8	21.1	1.6	***(+)	566.4	34.099	0.0372	0.0016
			2	8	7.1	0.8	***(-)	249.1	31.526	0.0286	0.0019
			3	8	1.5	0.7	***(-)	110.6	20.78	0.0136	0.0037
			5	8	0	0	***(-)	0	0	0	0
Endoconidiophora polonica	6	6	0	8	38.7	5.1		3509.2	139.809	0.011	0.0013
			1	8	17.9	3	***(-)	686.2	150.072	0.0264	0.002
			2	7	0.4	0.4	***(-)	34.1	30.546	0.0055	0.0064
			3	8	0	0	***(-)	0	0	0	0
			5	8	0	0	***(-)	0	0	0	0
Ophiostoma bicolor	6	8	0	8	71.6	6.3		3913.4	394.623	0.0184	0.0016
			1	8	13	2.5	***(-)	405.1	49.227	0.0321	0.0038
			2	8	9.4	3	***(-)	321.3	111.264	0.0295	0.0024
			3	8	7.1	1.9	***(-)	247.4	63.886	0.0287	0.0019
			5	8	1.6	1.5	***(-)	52.4	41.180	0.0214	0.0151
Grosmannia penicillata	6	8	0	8	26.9	3.6		2371.5	101.369	0.0113	0.0012
			1	8	23.9	1.8	ns	784	46.063	0.0305	0.0008
			2	8	11.1	1.4	***(-)	359.7	33.083	0.0309	0.0027
			3	8	3.9	0.9	***(-)	144.2	30.345	0.0267	0.002
			5	8	0	0	***(-)	0	0	0	0
Fusarium euwallaceae	5	7	0	8	90.1	10.5		2453	140.9	0.0367	0.0033
			1	8	92.1	11.2	ns	2123.6	206.982	0.0434	0.004
			2	8	83.7	9.7	ns	1757.1	200.436	0.0477	0.0023
			3	8	50.9	7.7	***(-)	1158.8	180.89	0.044	0.002
			5	8	10.4	3	***(-)	310.3	84.493	0.0336	0.0048

Ambrosiella hartigii	5	4	0	8	24.3	6.1		2382.8	366.111	0.0103	0.0026
			1	8	34.6	6.2	***(+)	2545.2	399.027	0.0136	0.0021
			2	8	17.6	3.5	**(-)	1708.6	246.778	0.0103	0.0013
			3	8	12	1.7	***(-)	1367.9	180.79	0.0088	0.001
			5	8	4.4	1	***(-)	495.65	146.733	0.0093	0.0018
Raffaelea canadensis	8	14	0	8	78.9	13.3		1456.9	178.767	0.0538	0.0025
			1	6	88.3	88.3	ns	1554.4	194.69	0.0567	0.0018
			2	8	62.3	62.3	*(-)	1222.3	427.909	0.0501	0.0051
			3	8	75.7	75.7	ns	1354.6	139.992	0.0558	0.0019
			5	8	27.9	27.9	***(-)	604.8	94.457	0.0459	0.0028
R. sulphurea	5	4	0	8	39	9.1		4666.4	202.519	0.008	0.0019
			1	8	44.4	9.9	ns	3425.9	438.281	0.013	0.0015
			2	7	14.6	8.7	***(-)	1469.5	712.242	0.009	0.0015
			3	8	9.1	6.5	***(-)	903.3	586.696	0.009	0.0017
			5	8	0.8	0.8	***(-)	102.7	96.904	0.004	0.0037
Chaetomium globosum	7	4	0	8	27.7	8		1739.1	418.448	0.0159	0.0017
			1	8	8	3.7	***(-)	362	157.962	0.022	0.0009
			2	8	3.5	3.1	***(-)	177.5	137.486	0.019	0.0022
			3	8	0.9	0.9	***(-)	58.3	48.797	0.012	0.0073
			5	8	0	0	***(-)	0	0	0	0
Beauveria bassiana	9	7	0	8	44	20.5		1030.5	888.811	0.0541	0.0135
			1	8	51.9	14.5	ns	1219.5	523.438	0.0455	0.0123
			2	8	44.5	28.1	ns	1082.6	699.225	0.0414	0.0056
			3	8	26.4	16.5	ns	804.7	552.697	0.0348	0.0068
			5	8	2.3	2	***(-)	98.4	94.495	0.0264	0.0123
Esteya vermicola	9	13	0	8	128	8.5		2669.6	152.859	0.048	0.0035
			1	7	167	11.1	**(+)	2188.3	137.5	0.076	0.0046
			2	8	144.3	13.3	ns	1591.2	118.544	0.091	0.0033
			3	8	103.3	13.6	*(-)	1344.3	233.985	0.078	0.0062
			5	8	53.8	15.8	***(-)	803.7	194.031	0.066	0.0066

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Chapter 4

Lehenberger M, Foh N, Göttlein A, Six D and Biedermann PHW. (in review) Nutrient-poor breeding substrates of ambrosia beetles are enriched with biologically important elements. *Frontiers in Microbiology,* submitted (February 2021).

Nutrient-poor breeding substrates of ambrosia beetles are enriched with biologically important elements

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Abstract

Fungus-farming within galleries in the xylem of trees has evolved independently in at least twelve lineages of weevils (Curculionidae: Scolytinae, Platypodinae) and one lineage of ship-timber beetles (Lymexylidae). Jointly these are termed ambrosia beetles because they actively cultivate nutritional “ambrosia fungi” as their main source of food. The beetles are obligately dependent on their ambrosia fungi as they provide them a broad range of essential nutrients ensuring their survival in an extremely nutrient-poor environment. While xylem is rich in carbon (C) and hydrogen (H), various elements essential for fungal and beetle growth, such as nitrogen (N), phosphorus (P), sulfur (S), potassium (K), calcium (Ca), magnesium (Mg), and manganese (Mn) are extremely low in concentration. Currently it remains untested how both ambrosia beetles and their fungi meet their nutritional requirements in this habitat.

Here, we aimed to determine for the first time if galleries of ambrosia beetles are generally enriched with elements that are rare in uncolonized xylem tissue and whether these nutrients are translocated to the galleries from the xylem by the fungal associates. To do so, we examined natural galleries of three ambrosia beetle species from three independently evolved farming lineages, *Xyleborinus saxesenii* (Scolytinae: Xyleborini), *Trypodendron lineatum* (Scolytinae: Xyloterini) and *Elateroides dermestoides* (Lymexylidae), that cultivate unrelated ambrosia fungi in the ascomycete orders Ophiostomatales, Microascales, and Saccharomycetales, respectively. Several elements, in particular Ca, N, P, K, Mg, Mn, and S, were present in high concentrations within the beetles’ galleries but available in only very low concentrations in the surrounding xylem. The concentration of elements was generally highest with *X. saxesenii*, followed by *T. lineatum* and *E. dermestoides*, which positively correlates with the degree of sociality and productivity of brood per

gallery. We propose that the ambrosia fungal mutualists are translocating essential elements through their hyphae from the xylem to fruiting structures they form on gallery walls. Moreover, the extremely strong enrichment observed suggests recycling of these elements from the feces of the insects, where bacteria and yeasts might play a role.

Keywords: Ambrosia beetle, ecological stoichiometry, microbiome, nutrients, macro- and micro-elements, element translocation

Introduction

In 1836, the monk Josef Schmidberger noticed a whitish layer in tunnel systems of some scolytine beetles within the sap- and heartwood (i.e., xylem) of his apple trees (Schmidberger, 1836). Observing the beetles in more detail he realized that this layer was nutritional and - in his view - was produced by the trees to nurture the beetles. Therefore, he termed this layer “ambrosia” (after the mythological perfect food of the gods). This layer was later found to be fungal fruiting structures (Hartig, 1844) and their beneficiaries came to be called “ambrosia beetles”. Even then it was regarded rather exceptional for insects to live within xylem, which is now known to be chemically well defended, nutritionally poor and extremely biased towards certain elements, particularly carbon (C), hydrogen (H), and oxygen (O) (Filipiak and Weiner, 2014; Pretzsch et al., 2014).

While most bark beetles (*sensu lato*) (Curculionidae: Scolytinae) colonizing the phloem or xylem of trees are well known for their facultative mutualism with fungi, ambrosia beetles (*sensu stricto*) among them have long been known for their obligate mutualisms with fungal symbionts (Kirkendall et al., 2015; Biedermann and Vega, 2020). In a broad sense, the term “ambrosia beetle” is used for all xylem-boring and fungus-farming beetles in the curculionid subfamilies Scolytinae (bark beetles) and Platypodinae (pinhole borers) as well as the Lymexylidae (ship-timber beetles)

(Neger 1908a). Ambrosia beetles are thus a polyphyletic group with at least 13 evolutionary independent origins of fungus farming (Biedermann and Vega, 2020). Common to all of them is their species-specific, obligate nutritional mutualism with filamentous fungi, which they transmit vertically in mycetangia (extracuticular spore-carrying organs; see Francke-Grosmann, 1956) and more or less actively farm in often social family groups (but Lymexylidae are solitary; (Francke-Grosmann, 1967; Beaver, 1989; Kirkendall et al., 2015; Hulcr and Stelinski, 2017; Biedermann and Vega 2020). Their mutualistic fungi are from at least five ascomycete (Ophiostomataceae, Ceratocystidaceae, Nectriaceae, Bionectriaceae, Saccharomycetaceae) and two basidiomycete (Peniophoraceae, Meruliaceae) (Hulcr and Stelinski, 2017; Biedermann and Vega, 2020) families. These species-specific mutualistic associates are highly adapted to their ambrosia beetle host. They strongly depend on the beetle for dispersal, are not capable of surviving outside the mutualism, produce nutrient-rich fruiting structures within the beetles' galleries, and are the sole or at least major food source for the beetles (Neger, 1908a, 1908b; Batra, 1963, 1966, 1967, 1973, 1985; Batra and Batra, 1967; Francke-Grosmann, 1966, 1967).

All herbivorous animals are confronted with a stoichiometric mismatch between the concentrations of elements required for growth and survival including nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu) and sodium (Na), and those found in their plant diet, which is rich in only C, H, and O (Sterner and Elser, 2002). For insects feeding within xylem this mismatch is extreme, as 99% of this tissue typically consists of C, H and O (Fengel and Wegener, 1989; Filipiak and Weiner, 2014) and elements such as N or P must be enriched by factors between 100 to 10,000 to support insect growth (Six and Elser, 2020). Hence, they have only two options (or a

combination of both); one is to develop very slowly and process massive amounts of plant material (see e.g. cerambycid or buprestid beetles) while the other is to supplement the diet with ectosymbiotic fungi that grow into the wood, and translocate and concentrate required elements for growth (Martin and Kukor, 1984; Buchner, 1928; Filipiak and Weiner, 2014). The latter process is assumed for ambrosia beetles that feed on their mutualistic ambrosia fungi (Biedermann and Vega, 2020), but whether rare elements are indeed absorbed by the fungi and translocated to the ambrosia layers made of asexual fruiting structures on the gallery walls has never been examined.

Ecological stoichiometry (hereafter termed ES) is a powerful approach which has the potential to answer such questions. In general, ES describes the movement and balance of elements in natural systems and how this is influenced by ecological interactions and processes (Sterner and Elser, 2002). Basically, ES offers the opportunity to examine the availability and limitations of elements within natural systems and thus, is a usefultool to gain insights into tropic relations such as the ecology and function of insect-microbe mutualisms. Nevertheless, only very few studies applied ES to these systems (Six and Elser, 2019, 2020). Six and Elser (2019) examined for the very first time within a bark beetle species, how fungal mutualists associated with *Dendroctonus brevicomis* concentrate and translocate elements such as P and N from the sapwood and phloem to the bark, where the beetles live and feed. To date, no studies applying ES to fungus-farming insect systems, such as ambrosia beetles, have been conducted and there have only been a few studies dealing with the nutrient flow in plant-fungus or animal-animal mutualisms (Kay et al., 2004; Johnson, 2010; Mariotte et al., 2017).

All organisms require a number of macro- and micro-nutrients to grow, develop, and reproduce. Insects feeding in plant tissues like wood are especially

challenged to acquire the nutrients they need and most, if not all, have symbioses with microbes that aid in alleviating imbalances (Buchner, 1928). For instance, N (needed for producing proteins and amino acids), S (needed for production of the amino acid methionine and cysteine), and P (needed for production of ribosomes, RNA, DNA, lipid layers, and ATP) (McDowell, 2003) occur in wood in very low concentrations (Fengel and Wegener, 1989; Filipiak et al., 2016). So wood-dwelling insects often make use of specific microbes to enrich their diet (Täyasu et al., 1994; Ayayee et al., 2014; Ulyshen, 2015; Filipiak et al., 2016; Filipiak and Weiner, 2017). Filamentous fungi among them may acquire the rare nutrients by growing far into plant tissues (Six and Elser, 2019), whereas some bacteria are capable of fixing atmospheric nitrogen (Peklo and Satava, 1949; Bridges, 1981; Täyasu et al., 1994; Six, 2003; Bleiker and Six, 2014; Filipiak and Weiner, 2014; Filipiak et al., 2016). Since ambrosia beetles do not feed outside their galleries, all nutrients needed for growth and reproduction for themselves and for their fungi must come from the surrounding xylem or the atmosphere (only N).

Here, we examined the availability and possible translocation of critical nutrients by fungi for three well-known and widespread ambrosia beetle species in three independently-evolved mutualisms of Xyleborini (Curculionidae: Scolytinae) with *Raffaelea* (Ophiostomatales: Ophiostomacaea) fungi, Xyloterini (Curculionidae: Scolytinae) with *Phialophoropsis* (Microascales: Ceratocystidaceae) fungi and Lymexylidae with *Alloascoidea* (Saccharomycetales: Saccharomycetaceae) filamentous yeasts: (i) The fruit-tree pinhole borer *Xyleborinus saxesenii* (Ratzeburg) (Xyleborini), (ii) the striped ambrosia beetle *Trypodendron lineatum* (Olivier) (Xyloterini), and (iii) the ship-timber beetle *Elateroides dermestoides* (syn. *Hylecoetus dermestoides*) (Linnaeus) (Lymexylidae). All three beetles are native to Europe but differ strongly in their social systems and farming behaviors. *Xyleborinus saxesenii* is

a cooperatively breeding species with a chamber-like gallery, where brood of all stages are found together with adult helpers, adult reproductives (mothers) and the farmed ambrosia fungi *Raffaelea sulphurea* (L.R. Batra) T.C. Harrington and *R. canadensis* (L.R. Batra) T.C. Harrington (Biedermann and Taborsky, 2011; Biedermann et al., 2013). This beetle shows little host preference, exhibits alloparental brood care and brother-sister inbreeding and can colonize the natal nest for more than two generations (Biedermann, 2010). *Trypodendron lineatum* prefers conifers and exhibits bi-parental care, but larvae develop within separate cradles with tunnel walls covered by *Phialophoropsis ferruginea* (Mathiesen-Käärik, 1953; Lehenberger et al., 2019; Mayers et al., 2020). Their galleries are active for only a single generation. No parent-offspring contact occurs in the host tree-generalist *E. dermestoides* (Coleoptera: Lymexylidae) whose larvae bore solitary tunnels and farm the yeast-like *Alloascoidea hylecoeti* (Neger) L.R. Batra (Francke-Grosmann, 1952; Batra and Francke-Grosmann, 1961; Batra, 1967). Developmental periods range from 1-2 months for the two scolytine species to 2-3 years for the lymexylid beetles (Francke-Grosmann, 1951; Popo and Thalenhorst, 1974; Biedermann et al. 2009). Differences in development may be partly explained by body size (*X. saxesenii* ~2 mm, *T. lineatum* ~3,5 mm, *E. dermestoides* 6-19 mm).

Here, we aimed to investigate if galleries of all three species are generally enriched with certain elements compared to uncolonized xylem indicating that the fungi may translocate and concentrate crucial nutritional elements from the surrounding xylem. We first visualized the elemental composition of xylem and gallery walls using scanning electron microscopy coupled with energy dispersive X-ray analysis (SEM-EDX). This technique has, to our knowledge, never been applied to an animal-microbe mutualism. It visualizes elements on biological surfaces but does not allow exact elemental quantification of such samples. Therefore, we then

quantified the elemental composition of both galleries and xylem using acid digestion and inductively coupled plasma optical emission spectroscopy (ICP-OES) which is widely used for examining woody samples (e.g. Huber et al., 2004; Górecka et al., 2006; Weis et al., 2009; Wellinger et al., 2012).

Materials and Methods

Collection and preparation of beetle galleries

We collected galleries of *X. saxesenii* (N = 13) and *E. dermestoides* (N = 1) in May 2019 from freshly dead beech (*Fagus sylvatica*) in the Bavarian Forest National Park (Neuschönau, Germany). Galleries of *T. lineatum* were collected from Norway spruce (*Picea abies*) in June 2019 in Mitterberg, Austria (N = 10). The galleries of the first two species still contained living insects, whereas *T. lineatum* galleries were from the previous season, but stored dry until dissection. For dissection of individual galleries, we used a chainsaw, an axe, and a spade chisel. Adult and immature specimen were either knocked out of the gallery or collected with fine tweezers. Immediately after opening a gallery, we carefully collected three different types of samples (each approx. 1 cm × 1 cm and 2-3mm deep) for elemental analysis: (**i**) gallery, (**ii**) surrounding xylem (about 1 cm next to the gallery in direction of wood fibers) and (**iii**) control xylem (at least 5 cm away from the gallery perpendicular to the direction of wood). Samples were stored in glass vials and galleries (for SEM-EDX) in paper bags at -20°C until they were processed.

To dry samples, we transferred them to a glass-desiccator filled with silica gel (without contact to the samples; Roth, Germany) and dried them for approximately three months. Galleries used for the SEM-EDX analysis were oven-dried for one week at 50°C within a slightly opened paper-bag ensuring the protection from any contamination and then stored within a glass-desiccator until they were analyzed.

Semi-quantification and visualization of elements by Energy-Dispersive X-ray Scanning-Electron-Microscopy (SEM-EDX)

SEM-EDX provides a semi-quantitative view of elemental compositions on the surface of samples (to a depth of ~2-3µm). It detects and identifies elements on the surface layer of a sample by detecting released element-specific radiation energy (e.g. Shindo and Oikawa, 2013; but see also Hudgins et al., 2003; De Vetter et al., 2006; Wang and Dibdiakova, 2014). However, to receive reliable data, it is important to minimize irregularities on the surface of an analyzed sample. Our flat sectioning of galleries reduced irregularities and allowed us to use it as a basis for the choice of elements to focus for the following ICP-OES absolute quantification. Three galleries from *X. saxesenii*, two from *T. lineatum* and one from *E. dermestoides* were used for SEM-EDX. With this design we aimed to test if certain elements are enriched within the beetles' galleries compared to the surrounding xylem and whether this is influenced by the direction of the fibers (fungi often grow along the wood fibers).

To prepare samples, we carefully opened each dried gallery and cut it to a size of 10 cm × 10 cm to fit onto an aluminum plate. To fix the position of each gallery on the plate, we used epoxy-resin (EPODEX, Germany). Afterwards, we carefully removed loose material like sawdust, beetle feces, and loose fungal mycelium from the surface of each gallery using filter-cleaned compressed air.

An EVO MA15 (Zeiss; Software: Esprit Version) scanning electron microscope (SEM) connected to an energy dispersive X-ray detector (EDX; Detector: Bruker XFlash 5010; Software: Bruker Quantax Esprit 1.9) within a semi-vacuum atmosphere was used for the measurements applying a primary energy of 20keV. Just before the measurements, we placed a few small pieces of aluminum foil onto our samples (outside the areas of interest), which helped us to navigate and locate

the areas of interest under the SEM. As we introduced this element to all samples used for SEM-EDX, we did not include it in our further analyses. A single measurement covered an area of about 600 µm × 800 µm. Multiple measurements from each gallery were taken along two lines, one in a vertical and the other in a horizontal direction to the direction of the wood fibers, covering both the inside ("gallery") as well as the outside ("surrounding xylem") of the beetle's gallery. For two measurements (one gallery each of *X. saxesenii* and of *T. lineatum*), we used the mapping function of the SEM-EDX, which allowed us to visualize the abundance of certain elements using different colors. Each gallery was exposed to an electron irradiation (20 keV) for approximately 10 min, in which we allowed the EDX-detector to identify element-specific radiation energies that were released by the sample.

Finally, we created line charts to visualize the presence of certain elements using ggplot in R (version 1.2.5033) with the packages "ggplot2" (Wickham, 2016) and "plotly" (Sievert, 2020). Colored lines (one color per element) were manually added and indicate the respective elements, while the length of each vertical line approximates the size of each peak (i.e., abundance of the element) with the exception of C, N and O which were either overabundant (C, O) or poorly quantifiable (N) using SEM-EDX.

Exact quantification of elements using Inductively Coupled Plasma Optic Emission Spectroscopy (ICP-OES) and an element analyzer

Here, we examined galleries from *T. lineatum* (N = 7 - 8; see Suppl. Table S1) and *X. saxesenii* (N = 9 - 10; see Suppl. Table S2) using acid digestion and ICP-OES (ARCOS by Spectro, Kleve Germany), while the C, N and H concentrations were determined by dry combustion in an element analyzer (*T. lineatum*: N = 5 – 8, see Suppl. Table S1; *X. saxesenii*: N = 9 – 10, see Suppl. Table S2) (vario EL III by

Elementar, Langenselbold Germany). The variation in the number of replicates is caused by few samples of insufficient size as well as by some extreme outliers (i.e., measurement errors, see below) which were removed prior to analyses.

The quantification was accomplished at the Professorship of Forest Nutrition and Water Resources (Technical University of Munich, Freising, Gemany). The dried samples were analyzed for their elemental composition according to German Forest Analytical Standards (König et al., 2005). Based on the received output of the SEM-EDX analysis, we quantified the following elements: P, K, S, Mg, Fe, Mn, Cu, Zn, Ca (after acid digestion using ICP-OES) as well as C, H and N (using an element analyzer). Approximately 60 mg per woody sample were digested with freshly distilled nitric acid (HNO3) at 160°C for 10h within quartz vessels using a high-pressure digestion apparatus (Seiff, Germany). The digest was diluted to 14 ml with distilled water and analyzed for element concentration by ICP-OES.

Since analytically conspicuous samples could not be checked (each sample was unique and the analysis is destructive), extreme outliers were removed from the total data set according to Tukey´s procedure (Tukey, 1977). The concentration of each investigated element per individual treatment was visualized with ggplot - boxplots in R (version 1.2.5033) using the package “ggplot2” (Wickham, 2016). Additionally, linear models (sqrt transformed data) were conducted to detect significant differences between the three different treatments (Gallery, Surrounding xylem, Control xylem) for each individual beetle species. Here, we used the R-packages “LMERConvenienceFunctions” (CRAN.R-project.org/package=LMERConvenienceFunctions), “multcomp” (Hothorn et al., 2008), “lsmeans” (Lenth, 2016) and “lme4” (Bates et al., 2015). Further, we calculated enrichment ratios for each examined element per treatment relative to control xylem as well as the molar ratios for C:P (carbon:phosphorus), C:N (nitrogen),

N:P, N:S (sulfur), N:Ca (calcium), N:K (potassium), N:Mg (magnesium), N:Mn (manganese), and N:Zn (zinc) for each of the three treatments

Results

Semi-quantification and visualization of elements by Energy-Dispersive X-ray Scanning-Electron-Microscopy (SEM-EDX)

The semi-quantitative SEM-EDX analysis revealed that all examined galleries (3 × *X. saxesenii*, [Fig. 1 - 2, Suppl. Fig. S1 - S2]; 2 × *T. lineatum*, [Fig. 3 - 4, Suppl. Fig. S3]; 1 × *E .dermestoides*, [Fig. 5]) were enriched with N, Mg, P, S, K, and Ca (in case of *E. dermestoides*, S appears to be absent within the analyzed gallery). Further, Mg could not be identified within one of the three galleries of *X. saxesenii* (see Fig. 1) using SEM-EDX, but the presences of this element within this specific gallery was proven by the EDX-Mapping function (see Fig. 2). Samples from uncolonized xylem had very low elemental compositions, which seemed to be independent of the direction of the wood fibers (i.e. horizontal vs. vertical measurements from the gallery in Fig. 1, 3, 5). Within uncolonized xylem, we mainly detected Ca, K, C and O (for *E. dermestoides*, Ca could not be detected outside the gallery), which appears to be also present within the galleries of all examined beetles. C and O are very abundant in all types of samples, so we did not include them in the EDX-mapping. We detected Mn only within the analyzed galleries of *X. saxesenii*. For *T. lineatum*, we examined both galleries and pupal chambers. Here, pupal chambers (Fig. 3 a & c; Suppl. Fig. S3 a & b) seemed to be generally enriched with P, S, Ca, K, and N, while Mg appears to be absent. The EDX-Mapping function supported our findings as visualized gallery tissues were enriched with S, N, Ca, P, and Mg for *X. saxesenii* (see Fig. 2) as well as with K, Ca, P, and N for *T. lineatum* (see Fig. 4)

relative to surrounding xylem. Raw data for the SEM-EDX analysis can be found in the supplementary material (see Suppl. Data S1 – S3).

Exact quantification of elements using Inductively Coupled Plasma Optic Emission Spectroscopy (ICP-OES) and an element analyzer

In a second step, we analyzed samples of 10 galleries of *X. saxesenii* and 8 galleries of *T. lineatum*, that were divided into the treatments (**i**) gallery, (**ii**) surrounding xylem, and (**iii**) control xylem. Generally, we confirmed our previous findings using quantitative ICP-OES and the element analyzer. We found significant incorporation of Ca ($p = 0.0005$, **i** to **ii**; $p = 0.0141$, **i** to **iii**), K, Mg, P, S, N ($p < 0.0001$, **i** to **ii** and **iii**), Mn ($p = 0.0066$, **i** to **ii**; $p = 0.0087$, **i** to **iii**), and Zn ($p = 0.0173$, **i** to **ii**) within galleries of *X. saxesenii* (see Fig. 6 – 7; Supplementary Table 1). Enrichment ratios of galleries relative to control xylem ranged from no enrichment of Fe (**i**: 0.96, **ii**: 0.91), Cu (**i**: 0.97, **ii**: 0.67), C (**i**: 1.0, **ii**: 1.0) and H (**i**: 1.0, **ii**: 1.0) to slight enrichment of Ca (**i**: 1.41, **ii**: 0.91), K (**i**: 2.19, **ii**: 1.02), Mg (**i**: 1.7, **ii**: 0.93), Mn (**i**: 1.48, **ii**: 1.01),, and Zn (**i**: 1.47, **ii**: 0.85), and to strong enrichments of P (**i**: 16.4, **ii**: 1.8), S (**i**: 3.31, **ii**: 1.01) and N (**i**: 4.27, **ii**: 1.28) (Supplementary Table 3). These enrichment ratios were also reflected by the C:N, C:P, and N:P ratios as well as all other ratios (N:S, N:Ca, N:K, N:Mg, N:Mn, N:Zn) (see Supplementary Table S5).

For *T. lineatum*, we did not analyze pupal chambers, but focused on the beetles´ main galleries, which were enriched with Ca ($p = 0.0281$, **i** to **ii**; $p = 0.0004$, **i** to **iii**), K ($p = 0.0149$, **i** to **iii**), Mg ($p = 0.0479$, **i** to **ii**; $p = 0.0026$, **i** to **iii**), P, S ($p < 0.0001$, **i** to **ii** and **iii**), and N ($p = 0.0005$, **i** to **ii**; $p = 0.0036$, **i** to **iii**) (see Fig. 8 – 9; Supplementary Table 2). Interestingly, the amount of H (see Fig. 9) was found to be higher in treatment **i** and **ii** compared to **iii** ($p = 0.0003$, **i** to **iii**; $p = 0.0004$, **ii** to **iii**). Enrichment ratios of galleries relative to control xylem ranged from no enrichment of

Fe (**i**: 0.86, **ii**: 1.16) and C (**i**: 0.99, **ii**: 0.99), to slight enrichments of Ca (**i**: 1.73, **ii**: 1.27), Cu (**i**: 1.87, **ii**: 0.61), , K (**i**: 1.68, **ii**: 1.26), Mg (**i**: 1.7, **ii**: 1.22), Mn (**i**: 2.5, **ii**: 2.32), S (**i**: 1.91, **ii**: 0.88), Zn (**i**: 1.38, **ii**: 1.17), and H (**i**: 1.17, **ii**: 1.15), and strong enrichments of P (**i**: 3.54, **ii**: 1.02) and N (**i**: 3.09, **ii**: 0.80) (Supplementary Table 4). Again enrichment ratios were well reflected by element ratios (C:N, C:P , N:P, N:S, N:Ca, N:K, N:Mg, N:Mn, N:Zn) (see Supplementary Table S5).

A majority of the elements were more abundant in galleries of *X. saxesenii*, except Ca, which was found in higher concentrations in galleries of *T. lineatum* (cf. y-axes in Figs. 6 - 9). This is striking, because some element ratios like C:P (36061.25 vs 29225.61), N:P (20.41 vs 8.3), N:S (10.93 vs. 6.22), N:Ca (8.76 vs. 1.5) and N:Zn (294.49 vs. 57.22), were much smaller in beech control xylem of *X. saxesenii* than spruce control xylem of *T. lineatum*. This is only partly explainable by the higher C:N ratio of spruce control xylem (3523.37 vs. 1766.57) (see Supplementary Table S5).

Discussion

Fungus-farming ambrosia beetles breed in xylem which is extremely poor in many essential nutrients and very much biased towards C, H, and O (Fengel and Wegener, 1989). It has been a long-standing question how the nutritional needs of the beetles are satisfied within this extreme habitat. Here, we show that the galleries of three, phylogenetically unrelated ambrosia beetles are enriched by several essential elements, out of which the enrichment ratios relative to xylem are most striking for P, S, and N. For example, the *Raffaelea* fungal mutualists of *X. saxesenii* are apparently able to greatly enrich their host's galleries with P by a factor of 16.4 relative to beech control xylem with a molar C:P ratio of only 36061.25, indicating high availability of P for the beetles.

Detailed measurements revealed that there is a sharp increase in elemental composition where the gallery borders the xylem (see Fig. 2 and Fig. 4). It appears

that the mutualistic, filamentous ambrosia fungi are translocating and concentrating essential elements from the surrounding xylem to their fruiting structures (i.e., conidiophores and conidia) on the gallery walls, which are then available to their beetle hosts as food. While N might be additionally enriched by bacterial fixation from the atmosphere (Bridges, 1981; Six, 2013), galleries are aerobic and make this possibility unlikely. No other elements are fixed from the atmosphere and thus translocation from outside of the gallery (from the xylem) would be required for such increases as we observed to occur. Translocation from the more nutrient-rich phloem is also, in principal, possible, but may be excluded by the finding that the enrichment of elements was generally lower in entrance tunnels of the galleries closer to the phloem of the colonized trees (see Supplementary Fig. 2). Yeasts and bacteria, which are ubiquitous associates within ambrosia beetle galleries (Beaver, 1989; Davis, 2015; Kirkendall et al., 2015; Hulcr et al., 2017), are unicellular organisms and thus are not capable of growing into and translocating elements from the xylem to the gallery, so we propose that enrichment is solely done by the (ambrosia) fungal symbionts.

If translocation of nutrients from the immediate surrounding of galleries by the mutualistic fungi occurs, we should find a depletion of elements near galleries but not in areas distant to galleries where fungi have not colonized the xylem. However, there was no detectable difference in elemental composition of xylem surrounding the galleries (assumed to be colonized by fungi) and control xylem located some distance from the galleries (assumed to be uncolonized) (Fig. 6 – 9). Even though the enrichment ratio of the surrounding xylem relative to the control xylem was in a few cases >1, the enrichment was not statistically significant for any micronutrient (Figs. 6 – 9; see also molar ratios in Supplementary Table S5). Qualitatively this finding was also supported by similar elemental compositions of presumably fungus-colonized vs.

uncolonized xylem samples taken in vertical vs. horizontal directions (with and against the grain) using SEM-EDX (Fig. 1 – 5 & Suppl. Fig. S1 – S3). Only H was significantly more abundant in surrounding xylem of *T. lineatum* galleries, which might indicate enzymatic cleavage of H-bonds by the *Phialophoropsis* fungal mutualists that are known to be potent degraders of xylan and cellulose (Lehenberger et al., 2019).

We have a few potential explanations why we did not detect such a pattern: First, ambrosia beetles (or their symbionts) may acquire nutrients independent of the xylem. However, apart from some symbiotic bacteria that might fix atmospheric N (Peklo and Satava, 1949; Bridges, 1981; Morales-Jiménez et al., 2009; Six, 2013), none of the other elements are fixed from the atmosphere and galleries are isolated from the external environment so we regard this explanation rather unlikely. Second, it may be that the fungi colonize much deeper into the xylem than previously thought. Given the low concentration of the elements in xylem (Fig. 6 -9, but see also Pretzsch et al., 2014), the fungi may need to colonize large areas of the xylem or even access more nutrient-rich parts of trees like phloem (Pretzsch et al., 2014; Ulbricht et al., 2016). Also, this option we regard rather unlikely because ambrosia beetle fungi are generally thought to be rather poor wood decomposers and may grow only a few mm inside the xylem (Francke-Grosmann, 1966). An exception may be *Phialophoropsis ferruginea* (associated with *T. lineatum*), which is depolymerizing major wood compounds such as cellulose and xylan (Lehenberger et al., 2019) and can grow up to 20 cm inside the xylem (Francke-Grosmann, 1966). But also for *T. lineatum*, there was no indication for stronger enrichment of the surrounding xylem. Regarding the translocation from phloem we did not see stronger enrichments of elements near the phloem in *X. saxesenii* entrance tunnels (see above). Third, the xylem serves as an elemental pipeline between roots and leaves (and *vice versa*) and within living trees

elements (in low concentrations) are constantly moving within the xylem (Matyssek et al., 2010). As ambrosia beetles are colonizing weakened or recently dead trees (Kirkendall et al., 2015), there might be still enough flux of elements through the xylem so that fungi might not necessarily need to colonize large areas of the xylem, but may capture essential elements out of the sap flow in the immediate surrounding of their galleries. It is questionable, however, if there is still enough flux or diffusion of elements in sap in wind-thrown or cut timber, which is also colonized by these beetles. Future studies on the fungal growth within the xylem are needed to answer whether the fungi, sap flow or diffusion are moving the elements towards the vicinity of the galleries.

The quantification analysis revealed striking differences regarding the concentration of certain elements between the galleries of *X. saxesenii* and *T. lineatum*. Generally, galleries of *X. saxesenii* contained much higher amounts of K, Mg, Mn, P, S, and N and the availability of especially N and P (based on molar ratios of C:P and C:N, see Supplementary Table S5) is much better than in galleries of *T. lineatum*. The only element that was found in higher amounts within the galleries of *T. lineatum* was Ca (N:Ca ratio of gallery: *T. lineatum* = 1.5, *X. saxesenii* = 8.76), which is known to affect the growth, branching, sporulation and/or virulence in fungi (Pitt and Ugalde, 1984; Lange and Peiter, 2020). Partly this is explainable by the higher abundances of some elements in the xylem of beech (i.e., control xylem from *X. saxesenii*; Fig. 6 and 7; Supplementary Table S5) than in the xylem of spruce (i.e., control xylem from *T. lineatum*; Fig. 8 and 9; Supplementary Table S5), with Ca being the only exception (~350 ppm in beech vs. ~550 ppm in spruce) (see also Pretzsch et al., 2014; Ulbricht et al., 2016). On the other hand, also elemental enrichment is strongest in *X. saxesenii*, followed by *T. lineatum* and then *E. dermestoides* (as we used a semi-quantitative pattern, we can only assume that this might be the case for

E. dermestoides based on the findings of the SEM-EDX analysis and the use of only one gallery). Interestingly, this pattern correlates with the social behavior and productivity (in terms of offspring numbers per gallery) of these species. Sociality is a derived trait in ambrosia beetles and cooperative breeding and eusociality are found in very few species (Kirkendall et al., 2015; Biedermann and Vega, 2020). The higher social species produce longer galleries and produce more offspring. Hence, more individuals spend longer periods inside the tree allowing more time for the fungi to move elements to their structures growing on gallery walls. *X. saxesenii* can occupy the same gallery with dozens of individuals over multiple generations for up to two years, whereas the other two species have only a single generation of a dozen individuals per gallery which lasts for only a few months (*T. lineatum*) or a single individual that develops over two years (*E. dermestoides*) (Francke-Grosmann, 1952, 1967, 1975; Popo and Thalenhorst, 1974; Biedermann and Taborsky, 2011; Biedermann et al., 2013).

Wood is finite in N, P, and other essential elements, which may also mean that the *Raffaelea* fungi of this *X. saxesenii* are particularly efficient to capture elements or may extend deeper into xylem than others (see option 2 or 3 above). This could, on the other hand, also be the precondition for *X. saxesenii* to evolve a social life with overlapping generations and long-term productivity. More work is needed to determine whether the stronger elemental enrichment in *X. saxesenii* is the cause or consequence of/for its social life. First, we need to know how deeply ambrosia fungi forage in xylem and whether different species have different foraging efficiencies that feedback to influence host fecundity. One important question that remains to be answered is whether different host trees influence elemental enrichment and beetle fitness. It would be interesting to investigate if the ambrosia fungi of *X. saxesenii* are able to provide equally high levels of enrichment when foraging within the less

nutritional xylem of spruce and how any differences may translate to effects on beetle fitness and sociality.

Apart from the questions raised above it would be interesting to investigate elemental accumulation from fungi growing in the galleries to the adult beetles and larvae (e.g. Six and Elser, 2020), which could give us insights into the specialization of ambrosia beetles to dead wood habitat. Furthermore, we need to determine how these elements may cycle within the gallery between beetles and their symbionts. In *X. saxesenii*, adults and larvae have been shown to press their feces against gallery walls, possibly to be recycled and further degraded by their fungal mutualists (De Fine Licht and Biedermann, 2012). Furthermore, there is unpublished data that uric acid (the major excretory product of insects) is one of the best nitrogen sources in media for culturing *Raffaelea sulphurea*, one of the two mutualists of *X. saxesenii* (R. Roeper, unpublished data; or see Norris (1979) for *Fusarium* ambrosia fungi).

Stable isotopes could be used to observe the nutrient-flow from woody tissue and fungi to the larvae and beetles (e.g. Hobbie et al., 1999; Griffith, 2004; Mayor et al., 2009). In the natural substrate this will be difficult, but a few ambrosia beetle species (e.g. *X. saxesenii*) can be reared within artificial media that may be supplemented with isotopes of interest (Gries and Sanders, 1984; Biedermann et al., 2009). Ecological stoichiometry is still in its infancy in insect-fungus mutualisms, but its application could help us to identify the nutrient flows upon which these systems are based. This may not only help us to understand their nutritional requirements and mechanisms of element capture, but might also help us to identify the key microbial players in these typically multipartite systems. Ultimately, ES studies conducted across a broad range of Scolytinae will allow us to develop a holistic model for the evolution of these mutualisms in what is arguably the most important group of insects affecting forest ecosystems worldwide.

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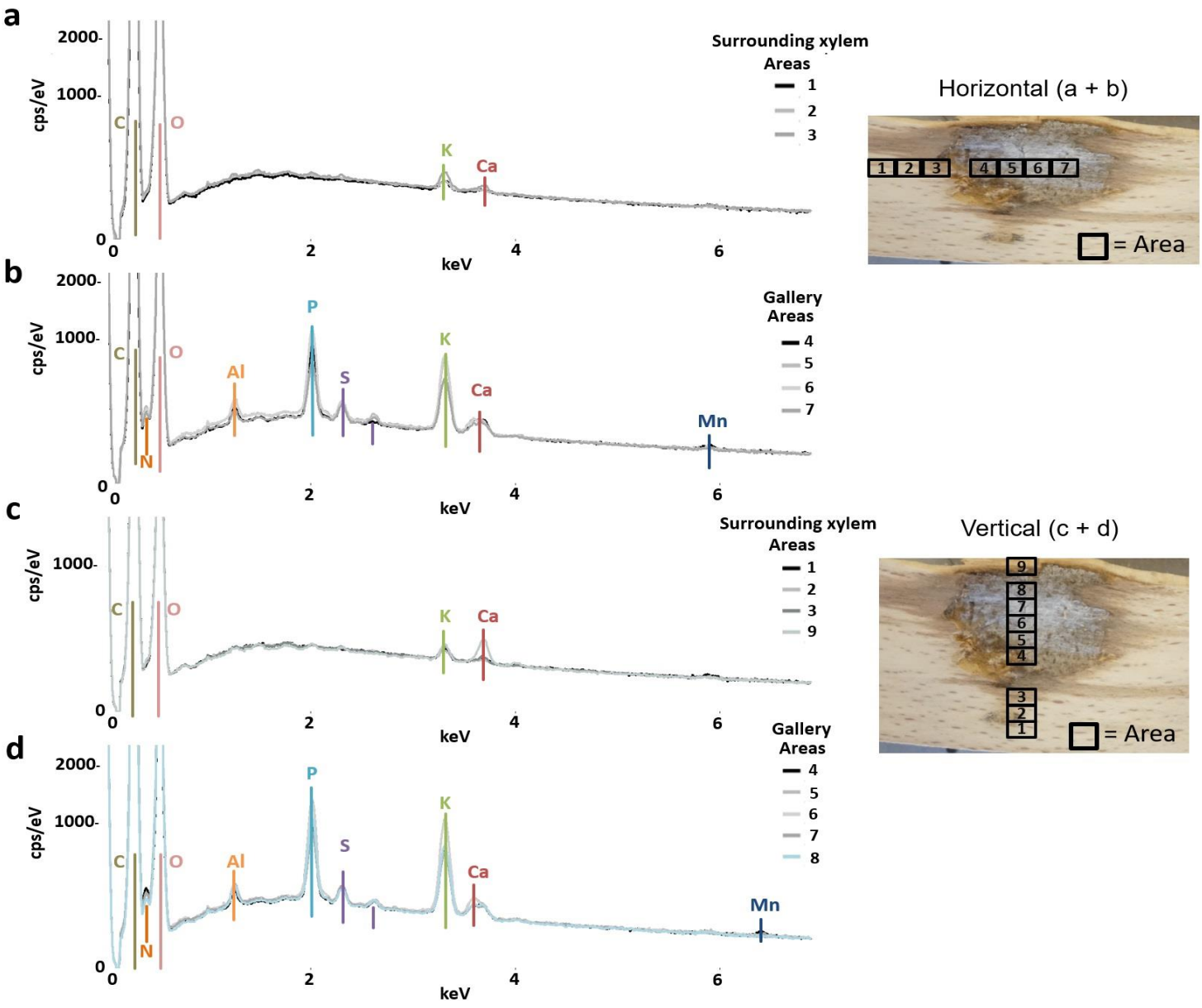

Figure 1: Elemental composition of gallery walls and xylem surrounding a gallery of *Xyleborinus saxesenii* (ID = X.sax13) using SEM-EDX. The gallery was examined in horizontal (a, b) and vertical (c, d) direction respective to wood grain; location and numbers of measured areas are given on the right. The Y-axis of each plot is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is the applied electricity in kilo-electronvolt (keV).

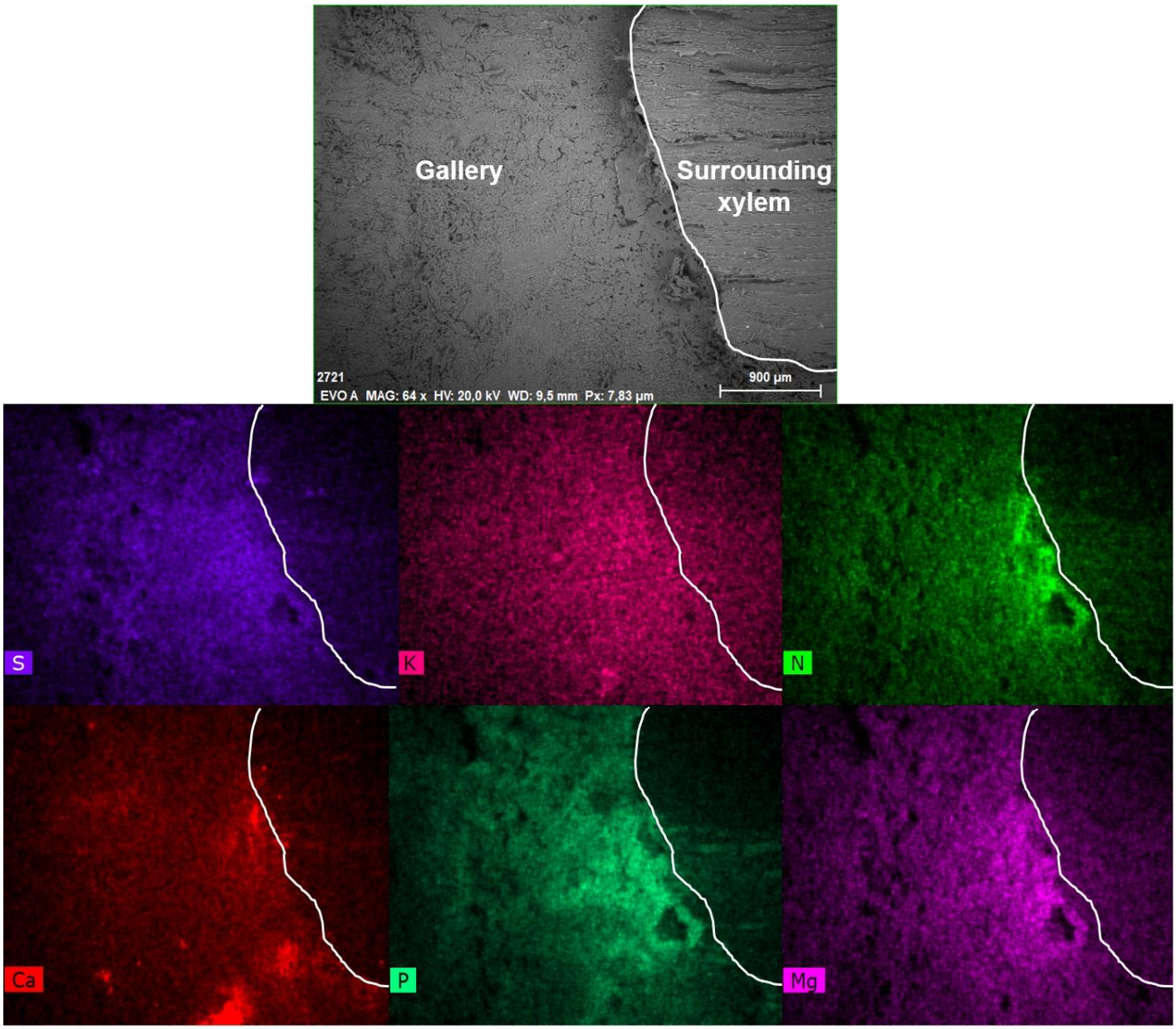

Figure 2: Visualization of elements within a gallery of *Xyleborinus saxesenii* (ID = X.sax2) using the mapping function of the SEM-EDX. The SEM image on top (EVO A) is the inside of the gallery (left) and the surrounding xylem (right, starting from the ridge). The border from gallery to surrounding xylem was indicated with a white line in each image. Scale bar and magnification (64 ×) are given at the bottom of the image. The intensity of the colors in the images below reflect the abundance of the elements sulfur (S), potassium (K), nitrogen (N), calcium (Ca), phosphorus (P), and magnesium(Mg).

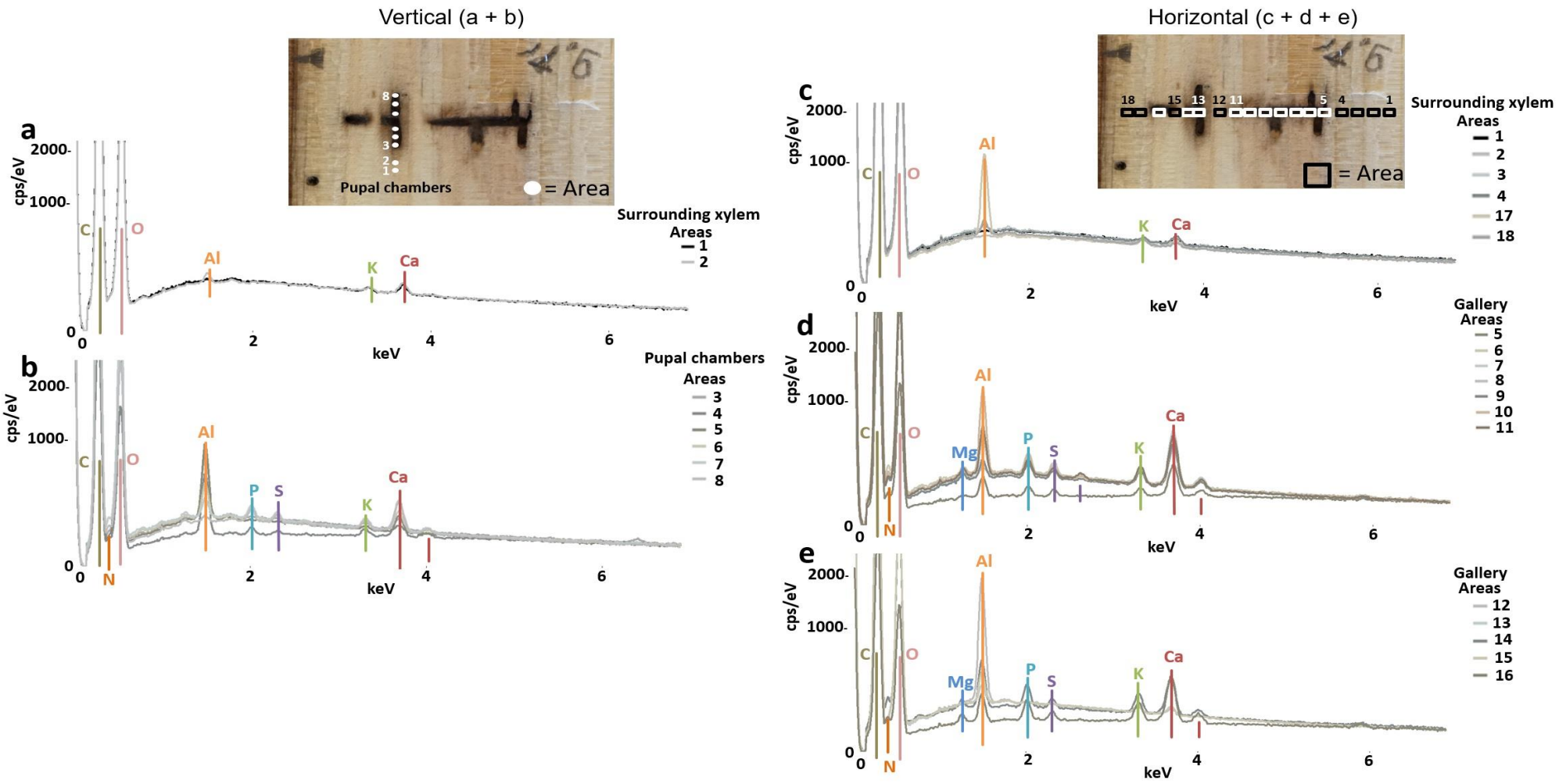

Figure 3: Elemental composition of gallery walls, pupal chambers and xylem surrounding a gallery of *Trypodendron lineatum* (ID = T.lin9) using SEM-EDX. Pupal chambers were examined in vertical (a – b) direction, while the gallery was examined in horizontal (c – e) direction, respective to wood grain; location and numbers of measured areas are given in the photos on top. The Y-axis of each plot is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is the applied electricity in kilo-electronvolt (keV).

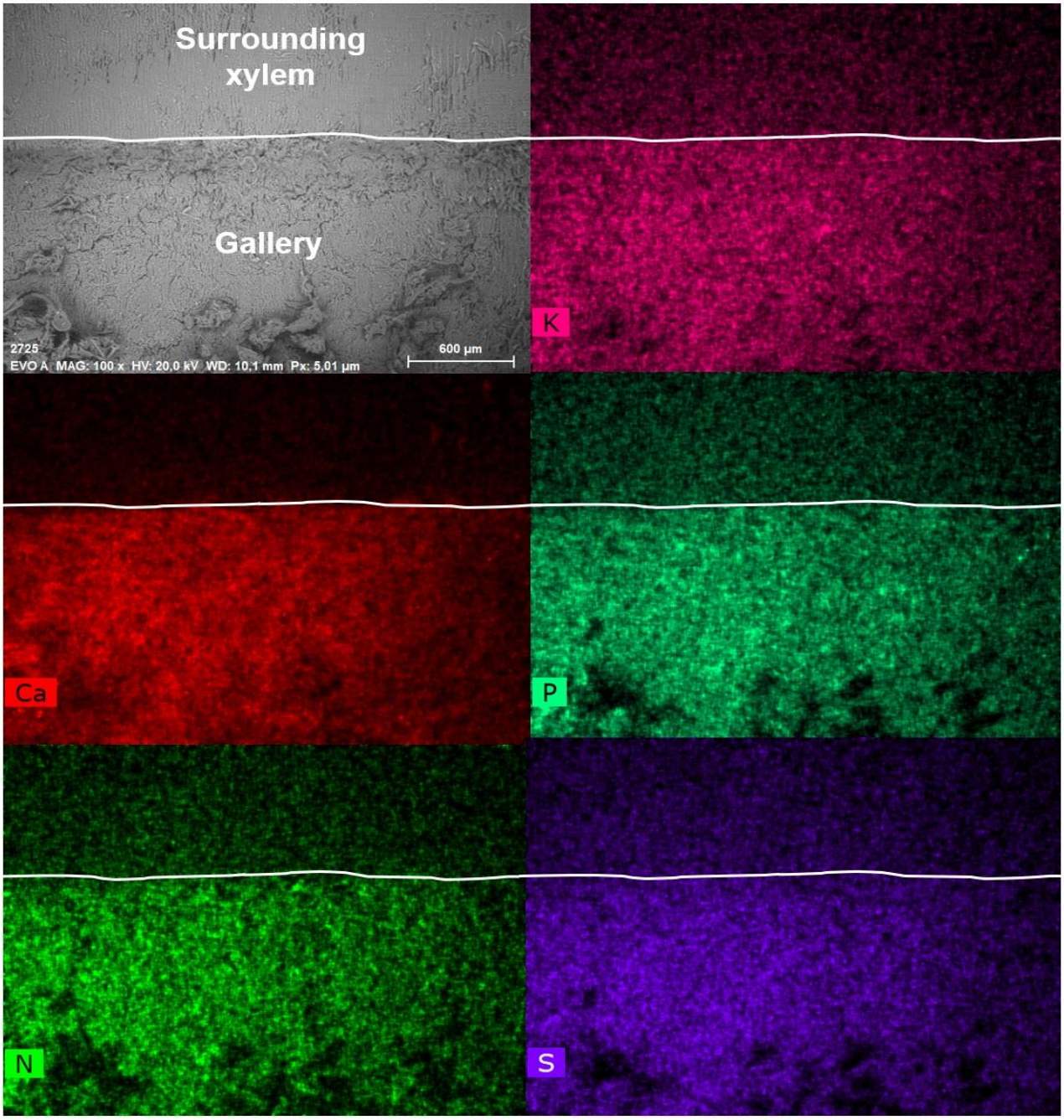

Figure 4: Visualization of elements within a gallery of *Trypodendron lineatum* (ID = T.lin9) using the mapping function of the SEM-EDX. The SEM image on the top left (EVO A) shows the inside of the gallery (bottom) and the surrounding xylem (top, starting from the ridge). The border from gallery to surrounding xylem was indicated with a white line in each image. Scale bar and magnification (100 ×) are given at the bottom of the image. The intensity of the colors in the other images reflect the abundance of the elements potassium (K), calcium (Ca), phosphorus (S), nitrogen (N), and sulfur (S).

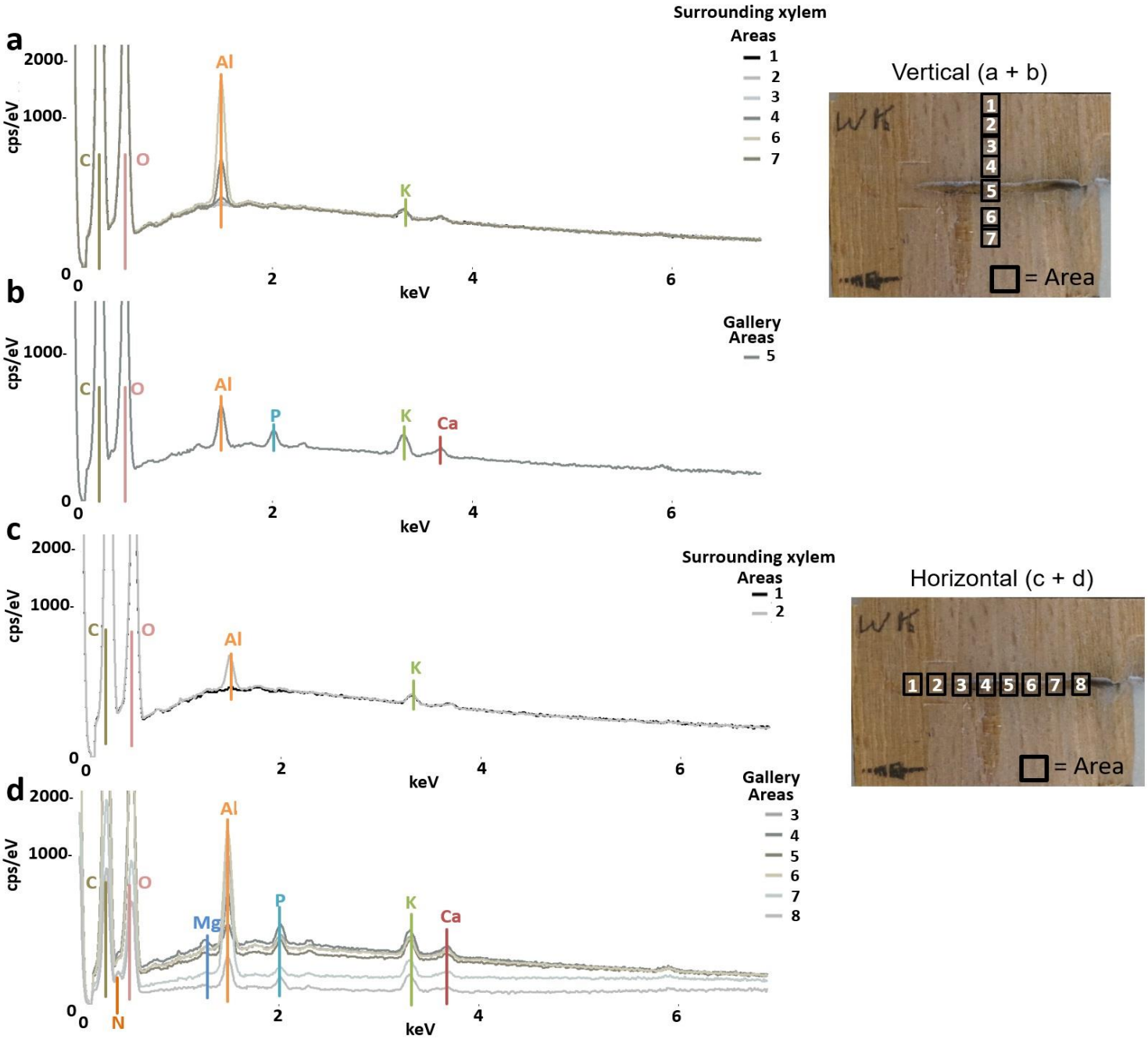

Figure 5: Elemental composition of gallery walls and xylem surrounding a gallery of *Elateroides dermestoides* (ID = HD) using SEM-EDX. The gallery was examined in vertical (a, b) and horizontal (c, d) direction respective to wood grain; location and numbers of measured areas are given in the photos on the right. The Y-axis of each plot is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is the applied electricity in kilo-electronvolt (keV).

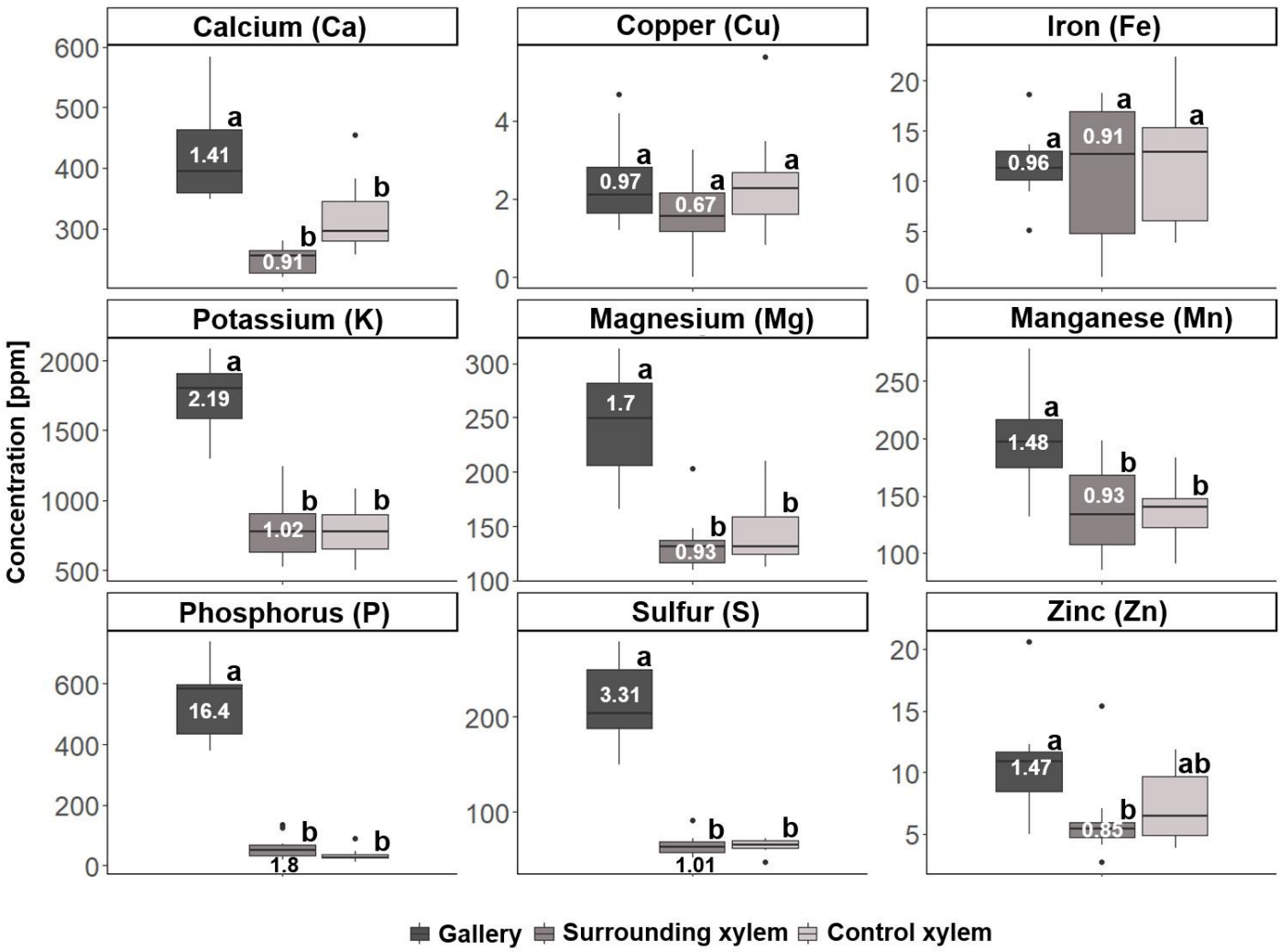

Figure 6: Concentrations of the different elements for the three types of samples taken from galleries of *Xyleborinus saxesenii* (N = 9 - 10 per group): (i) gallery, (ii) xylem next to gallery and (iii) control xylem. Elemental abundances are given in parts per million (ppm). Different letters next to boxplots (a, b) indicate significant differences between the respective group per individual element. Enrichment ratios (white numbers) for treatment i and ii were added to boxplots for each element. For P and S, enrichment rations were indicated below the boxplot (black numbers).

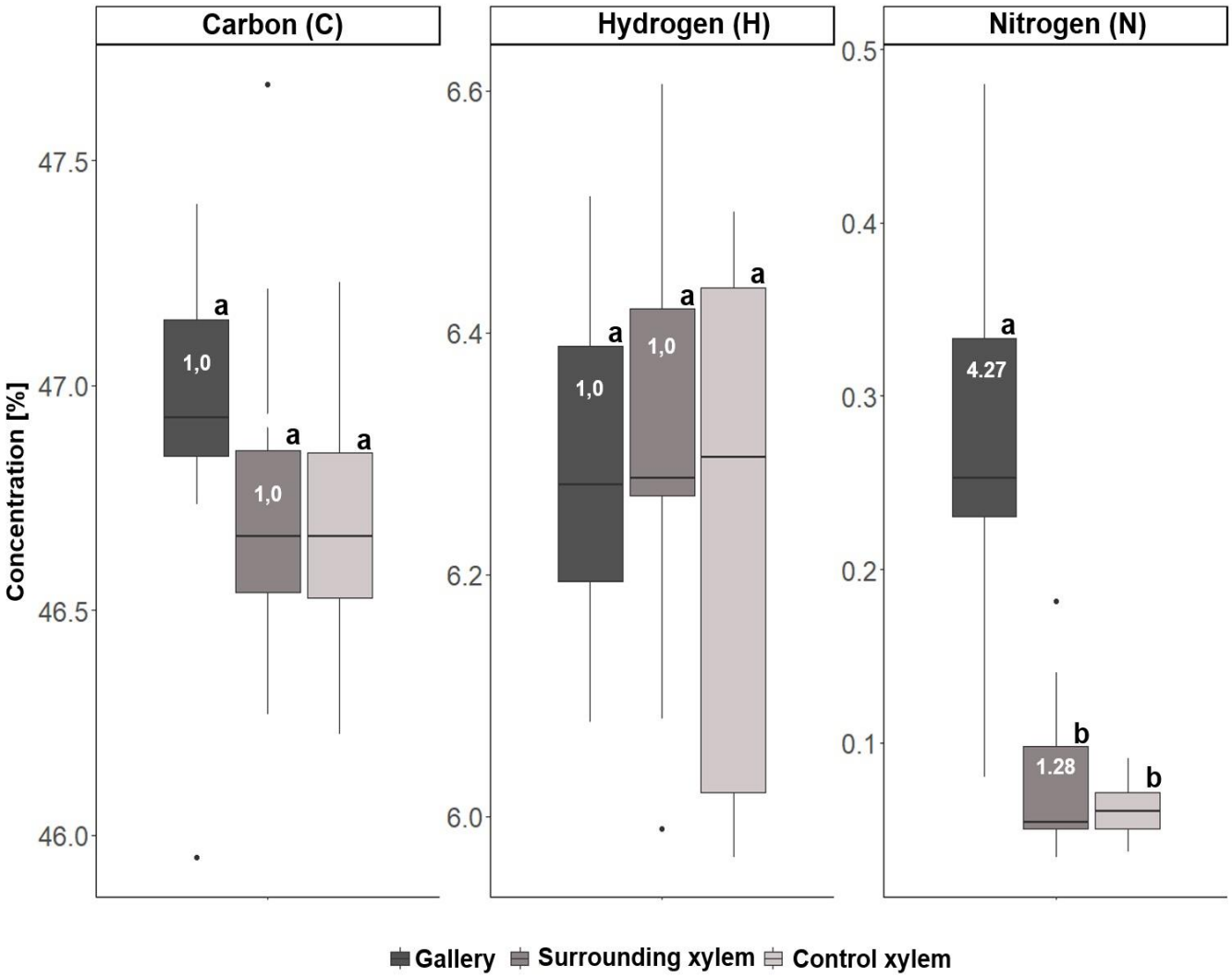

Figure 7: Concentrations of carbon (C), hydrogen (H), and nitrogen (N) for the three types of samples taken from *Xyleborinus saxesenii* (N = 9 - 10 per group): (i) gallery, (ii) xylem next to gallery and (iii) control xylem. Different letters next to boxplots (a, b) indicate significant differences between the respective group per individual element. Enrichment ratios (white numbers) for treatment i and ii were added to boxplots for each element.

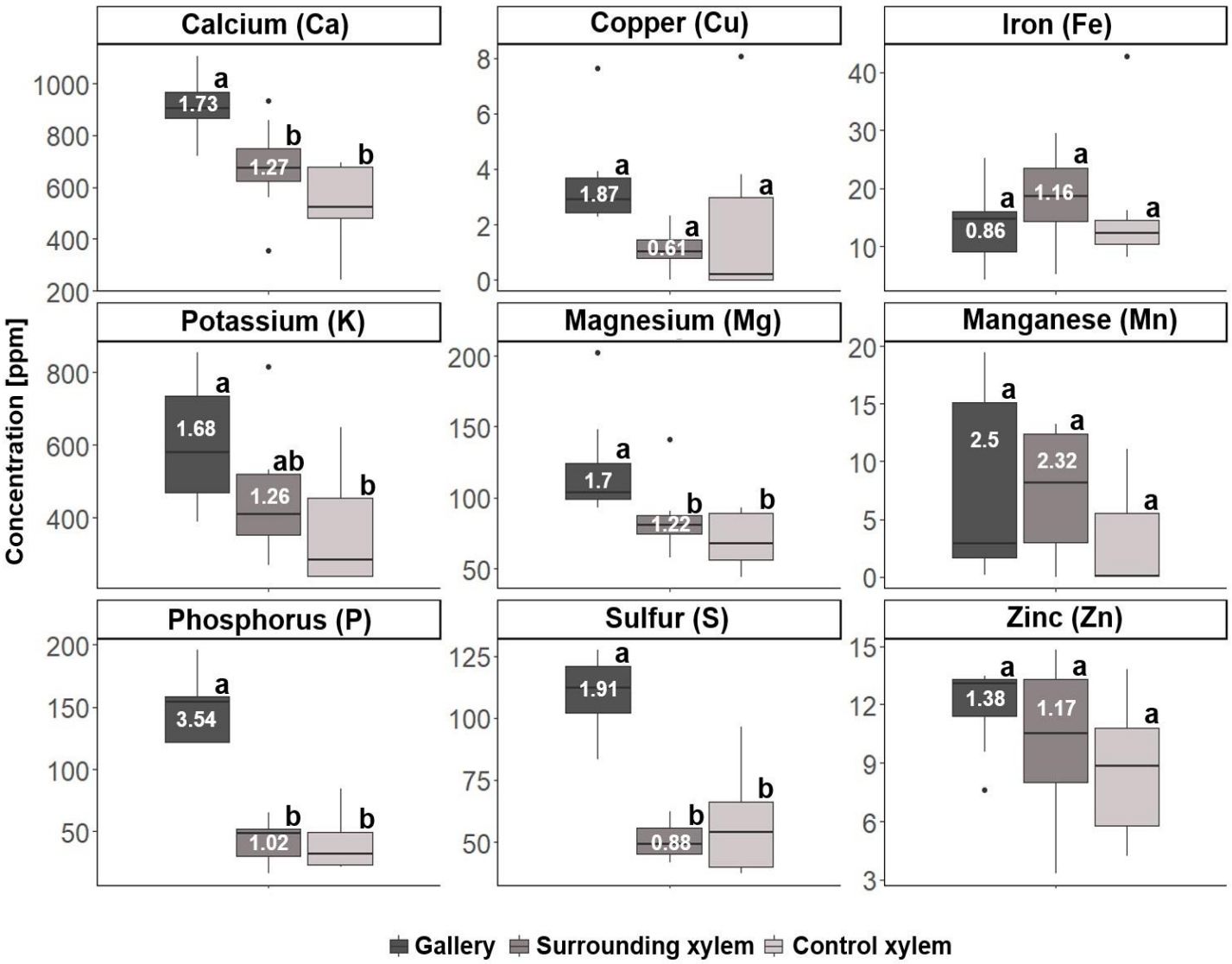

Figure 8: Concentrations of the different elements for the three types of samples taken from *Trypodendron lineatum* (N = 7 - 8 per group): (i) gallery, (ii) xylem next to gallery and (iii) control xylem. Elemental abundances are given in parts per million (ppm). Different letters next to boxplots (a, b) indicate significant differences between the respective group per individual element. Enrichment ratios (white numbers) for treatment i and ii were added to boxplots for each element.

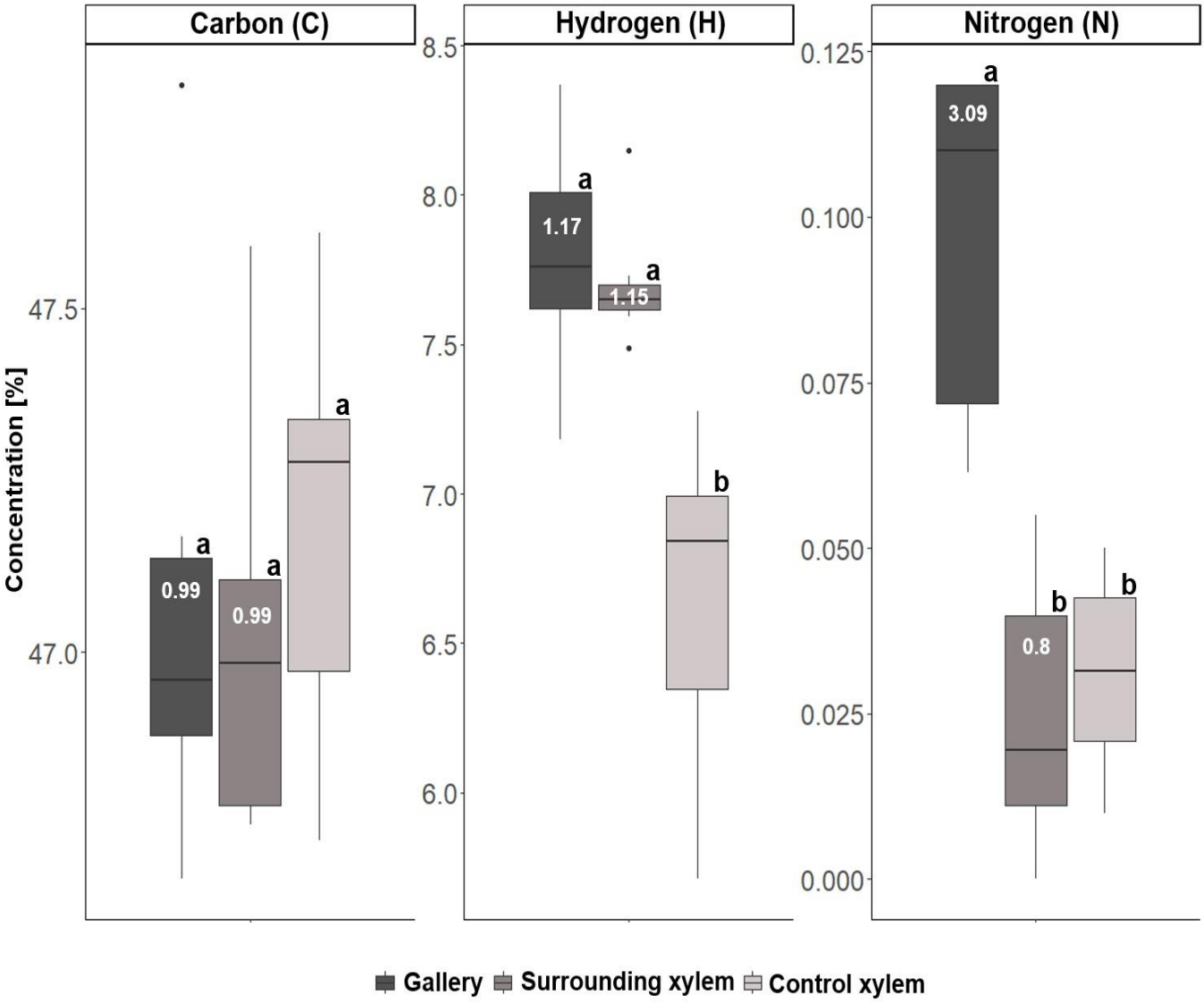

Figure 9: Concentrations of carbon (C), hydrogen (H), and nitrogen (N) the three types of samples taken from *Trypodendron lineatum* (N = 5 – 8 per group): (i) gallery, (ii) xylem next to gallery and (iii) control xylem. Different letters next to boxplots (a, b) indicate significant differences between the respective group per individual element. Enrichment ratios (white numbers) for treatment i and ii were added to boxplots for each element.

Annex – Supplementary Figures

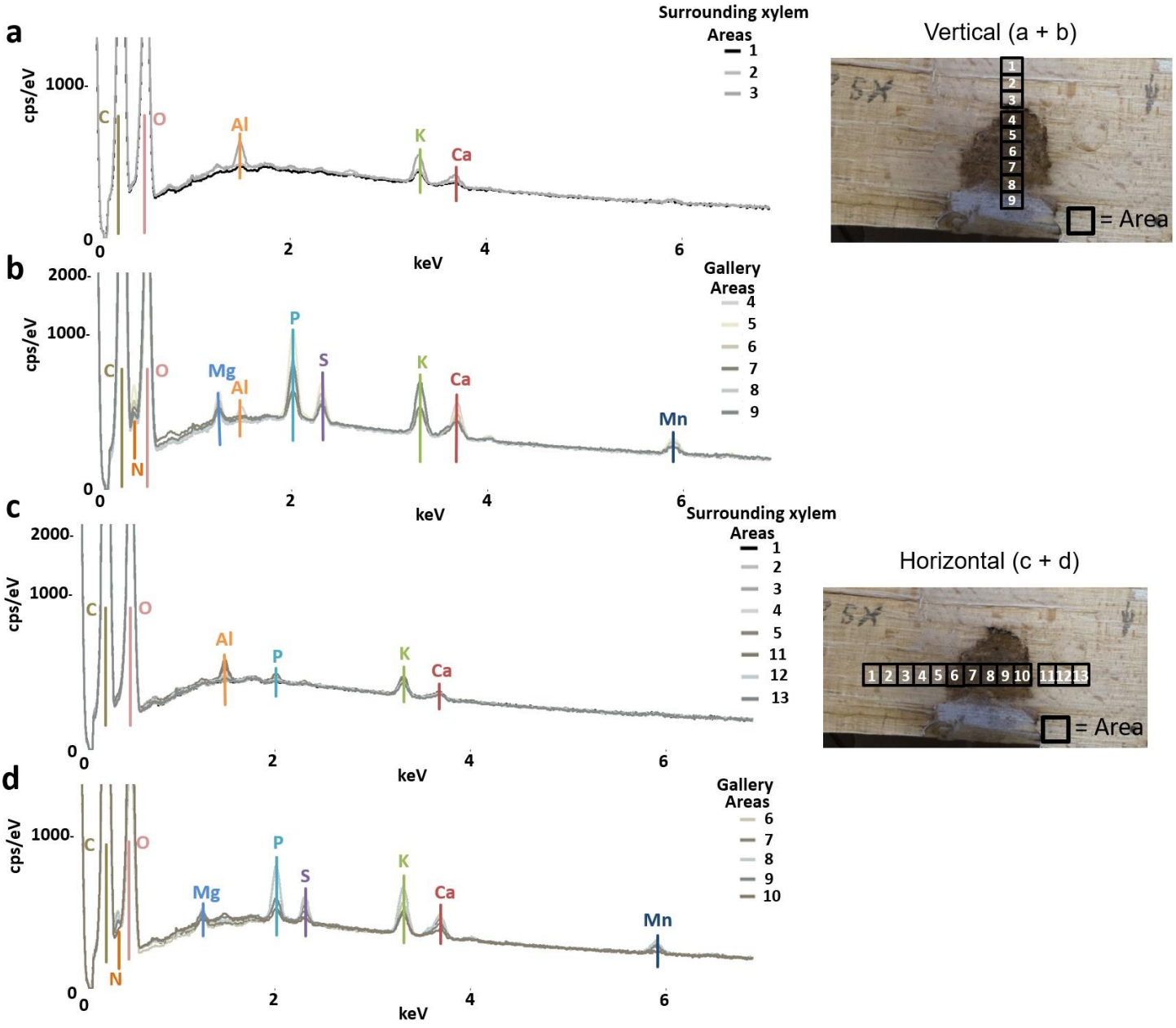

Figure S1: Elemental composition of gallery walls and xylem surrounding a gallery of *Xyleborinus saxesenii* (ID = X.sax2) using SEM-EDX. The gallery was examined in vertical (a, b) and horizontal (c, d) direction respective to wood grain; location and numbers of measured areas are given in the photos on the right. The Y-axis of each plot is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is the applied electricity in kilo-electronvolt (keV).

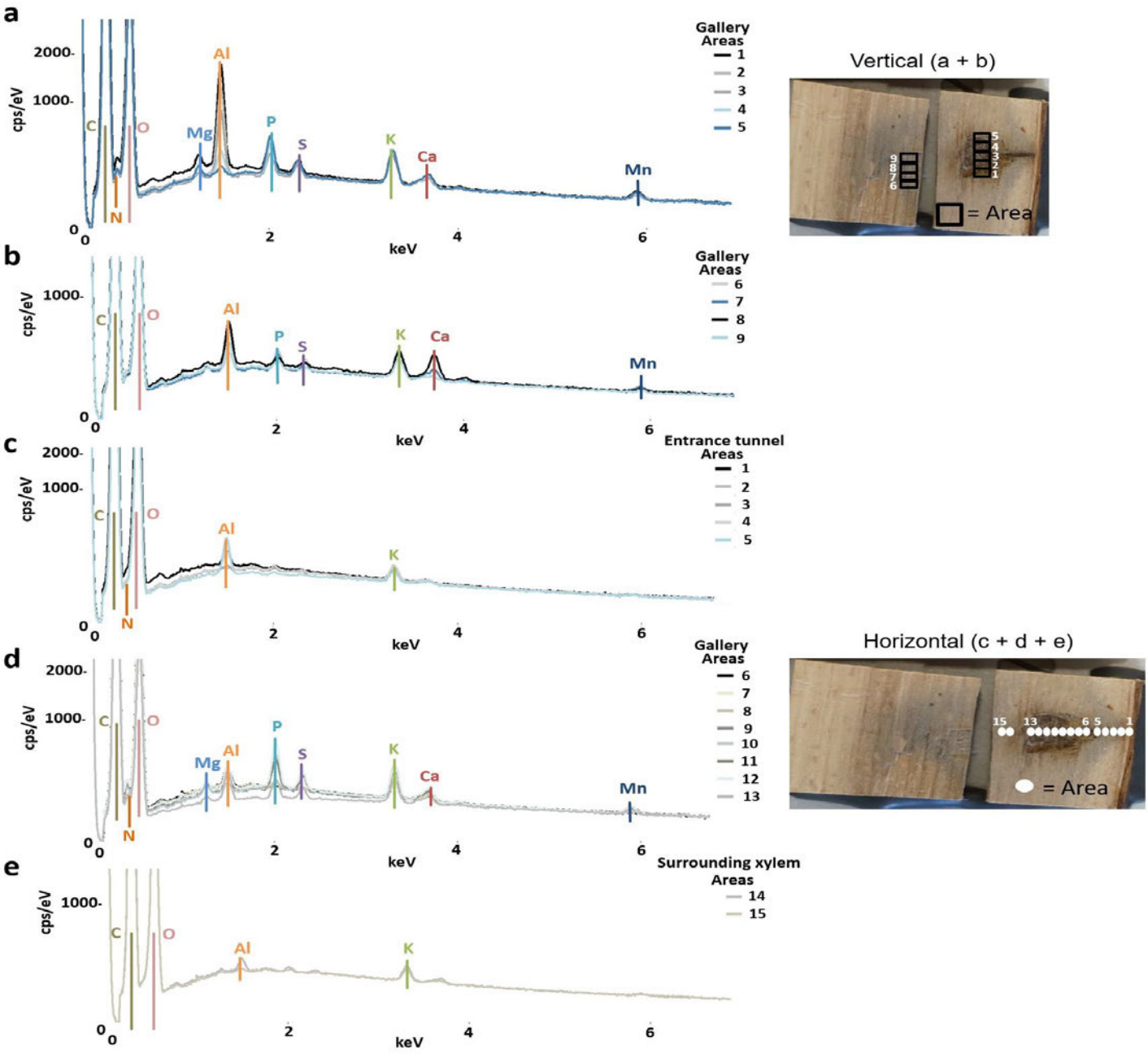

Figure S2: Elemental composition of gallery walls, entrance tunnel and xylem surrounding a gallery of *Xyleborinus saxesenii* (ID = X.sax3) using SEM-EDX. The gallery was examined in vertical (a, b) and horizontal (c – e) direction respective to wood grain; location and numbers of measured areas are given in the photos on the right. The Y-axis of each plots is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is showing the applied electricity in kilo-electronvolt (keV).

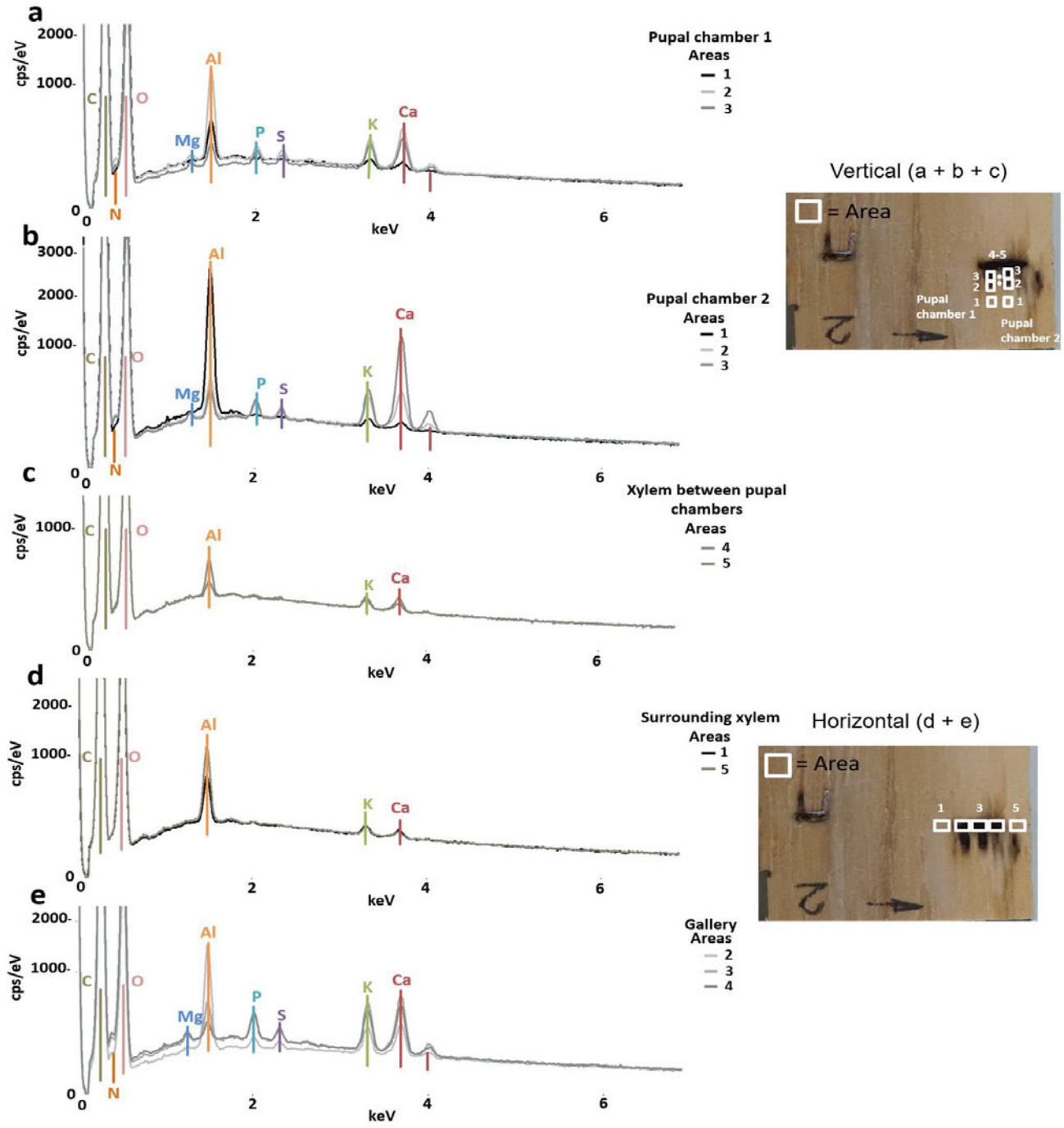

Figure S3: Elemental composition of gallery walls, pupal chambers and xylem surrounding a gallery of *Trypodendron lineatum* (ID = T.lin2) using SEM-EDX. Pupal chambers and surrounding xylem were examined in vertical (a - c) direction. The gallery was examined in horizontal (d – e) direction; location and numbers of measured areas are given in the photos on the right. The Y-axis of each plots is counts per second (cps) per electronvolt (eV) of each element, while the X-axis is the applied electricity in kilo-electronvolt (keV).

Annex – Supplementary Tables

Table S1: Overview of replicates (N) per element and treatment, and statistical differences between the three types of samples for *Trypodendron lineatum*: ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$.

Element	Treatment	Number of replicates (N)	Tested combination	Statistics (p)
Ca	Gallery	8	Gallery vs. Surrounding xylem	**0.0281 (*)**
Ca	Surrounding xylem	8	Gallery vs. Control xylem	**0.0004 (***)**
Ca	Control xylem	8	Surrounding xylem vs. Control xylem	0.1662 (ns)
Cu	Gallery	8	Gallery vs. Surrounding xylem	0.0756 (ns)
Cu	Surrounding xylem	8	Gallery vs. Control xylem	0.0667 (ns)
Cu	Control xylem	8	Surrounding xylem vs. Control xylem	0.9690 (ns)
Fe	Gallery	8	Gallery vs. Surrounding xylem	0.5086 (ns)
Fe	Surrounding xylem	8	Gallery vs. Control xylem	08914 (ns)
Fe	Control xylem	8	Surrounding xylem vs. Control xylem	0.7837 (ns)
K	Gallery	8	Gallery vs. Surrounding xylem	0.1855 (ns)
K	Surrounding xylem	8	Gallery vs. Control xylem	**0.0149 (*)**
K	Control xylem	8	Surrounding xylem vs. Control xylem	0.4337 (ns)
Mg	Gallery	8	Gallery vs. Surrounding xylem	**0.0479 (*)**
Mg	Surrounding xylem	8	Gallery vs. Control xylem	**0.0026 (**)**
Mg	Control xylem	8	Surrounding xylem vs. Control xylem	0.4134 (ns)
Mn	Gallery	7	Gallery vs. Surrounding xylem	1.0 (ns)
Mn	Surrounding xylem	7	Gallery vs. Control xylem	0.3056 (ns)
Mn	Control xylem	7	Surrounding xylem vs. Control xylem	0.3066 (ns)
P	Gallery	7	Gallery vs. Surrounding xylem	**<.0001 (***)**
P	Surrounding xylem	8	Gallery vs. Control xylem	**<.0001 (***)**
P	Control xylem	8	Surrounding xylem vs. Control xylem	0.9709 (ns)
S	Gallery	7	Gallery vs. Surrounding xylem	**<.0001 (***)**
S	Surrounding xylem	8	Gallery vs. Control xylem	**<.0001 (***)**
S	Control xylem	8	Surrounding xylem vs. Control xylem	0.6843 (ns)
Zn	Gallery	8	Gallery vs. Surrounding xylem	0.4810 (ns)
Zn	Surrounding xylem	8	Gallery vs. Control xylem	0.1353 (ns)
Zn	Control xylem	8	Surrounding xylem vs. Control xylem	0.6875 (ns)
C	Gallery	6	Gallery vs. Surrounding xylem	0.5705 (ns)
C	Surrounding xylem	8	Gallery vs. Control xylem	0.7993 (ns)
C	Control xylem	8	Surrounding xylem vs. Control xylem	0.1963 (ns)
H	Gallery	6	Gallery vs. Surrounding xylem	0.9385 (ns)
H	Surrounding xylem	7	Gallery vs. Control xylem	**0.0003 (***)**
H	Control xylem	7	Surrounding xylem vs. Control xylem	**0.0004 (***)**
N	Gallery	5	Gallery vs. Surrounding xylem	**0.0005 (***)**
N	Surrounding xylem	8	Gallery vs. Control xylem	**0.0036 (**)**
N	Control xylem	7	Surrounding xylem vs. Control xylem	0.6177 (ns)

Table S2: Overview of replicates (N) per element and treatment, and statistical differences between the three types of samples for *Xyleborinus saxesenii*: ns - $p > 0.05$, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$.

Element	Treatment	Number of replicates (N)	Tested combination	Statistics (p)
Ca	Gallery	9	Gallery vs. Surrounding xylem	**0.0005 (***)**
Ca	Surrounding xylem	9	Gallery vs. Control xylem	**0.0141 (*)**
Ca	Control xylem	10	Surrounding xylem vs. Control xylem	0.3609 (ns)
Cu	Gallery	10	Gallery vs. Surrounding xylem	0.2482 (ns)
Cu	Surrounding xylem	10	Gallery vs. Control xylem	0.9985 (ns)
Cu	Control xylem	9	Surrounding xylem vs. Control xylem	0.2445 (ns)
Fe	Gallery	10	Gallery vs. Surrounding xylem	0.8158 (ns)
Fe	Surrounding xylem	10	Gallery vs. Control xylem	0.9986 (ns)
Fe	Control xylem	10	Surrounding xylem vs. Control xylem	0.8428 (ns)
K	Gallery	10	Gallery vs. Surrounding xylem	**<.0001 (***)**
K	Surrounding xylem	10	Gallery vs. Control xylem	**<.0001 (***)**
K	Control xylem	10	Surrounding xylem vs. Control xylem	0.9946 (ns)
Mg	Gallery	9	Gallery vs. Surrounding xylem	**<.0001 (***)**
Mg	Surrounding xylem	10	Gallery vs. Control xylem	**<.0001 (***)**
Mg	Control xylem	10	Surrounding xylem vs. Control xylem	0.7659 (ns)
Mn	Gallery	9	Gallery vs. Surrounding xylem	**0.0066 (**)**
Mn	Surrounding xylem	9	Gallery vs. Control xylem	**0.0087 (**)**
Mn	Control xylem	10	Surrounding xylem vs. Control xylem	0.6907 (ns)
P	Gallery	10	Gallery vs. Surrounding xylem	**<.0001 (***)**
P	Surrounding xylem	10	Gallery vs. Control xylem	**<.0001 (***)**
P	Control xylem	10	Surrounding xylem vs. Control xylem	0.1976 (ns)
S	Gallery	10	Gallery vs. Surrounding xylem	**<.0001 (***)**
S	Surrounding xylem	10	Gallery vs. Control xylem	**<.0001 (***)**
S	Control xylem	10	Surrounding xylem vs. Control xylem	0.9990 (ns)
Zn	Gallery	10	Gallery vs. Surrounding xylem	**0.0173 (*)**
Zn	Surrounding xylem	9	Gallery vs. Control xylem	0.0928 (ns)
Zn	Control xylem	10	Surrounding xylem vs. Control xylem	0.6875 (ns)
C	Gallery	10	Gallery vs. Surrounding xylem	0.6734 (ns)
C	Surrounding xylem	10	Gallery vs. Control xylem	0.3893 (ns)
C	Control xylem	10	Surrounding xylem vs. Control xylem	0.8814 (ns)
H	Gallery	10	Gallery vs. Surrounding xylem	0.9892 (ns)
H	Surrounding xylem	9	Gallery vs. Control xylem	0.8712 (ns)
H	Control xylem	9	Surrounding xylem vs. Control xylem	0.8076 (ns)
N	Gallery	10	Gallery vs. Surrounding xylem	**<.0001 (***)**
N	Surrounding xylem	10	Gallery vs. Control xylem	**<.0001 (***)**
N	Control xylem	10	Surrounding xylem vs. Control xylem	0.8040 (ns)

Table S3: Enrichment ratios (ER) for each examined element based on the relative proportion of the means of the two treatments “Gallery” and “Surrounding xylem” to “Control xylem” for the samples of *Trypodendron lineatum*.

Element	**Treatment**	**ER**
Ca	Gallery	1.73
Ca	Surrounding xylem	1.27
Cu	Gallery	1.87
Cu	Surrounding xylem	0.61
Fe	Gallery	0.86
Fe	Surrounding xylem	1.16
K	Gallery	1.68
K	Surrounding xylem	1.26
Mg	Gallery	1.7
Mg	Surrounding xylem	1.22
Mn	Gallery	2.5
Mn	Surrounding xylem	2.32
P	Gallery	3.54
P	Surrounding xylem	1.02
S	Gallery	1.91
S	Surrounding xylem	0.88
Zn	Gallery	1.38
Zn	Surrounding xylem	1.17
C	Gallery	0.99
C	Surrounding xylem	0.99
H	Gallery	1.17
H	Surrounding xylem	1.15
N	Gallery	3.09
N	Surrounding xylem	0.8

Table S4: Enrichment ratios (ER) for each examined element based on the relative proportion of the means of the two treatments “Gallery” and “Surrounding xylem” to “Control xylem” for the samples of *Xyleborinus saxesenii*.

Element	**Treatment**	**ER**
Ca	Gallery	1.41
Ca	Surrounding xylem	0.91
Cu	Gallery	0.97
Cu	Surrounding xylem	0.67
Fe	Gallery	0.96
Fe	Surrounding xylem	0.91
K	Gallery	2.19
K	Surrounding xylem	1.02
Mg	Gallery	1.7
Mg	Surrounding xylem	0.93
Mn	Gallery	1.48
Mn	Surrounding xylem	1.01
P	Gallery	16.4
P	Surrounding xylem	1.8
S	Gallery	3.31
S	Surrounding xylem	1.01
Zn	Gallery	1.47
Zn	Surrounding xylem	0.85
C	Gallery	1
C	Surrounding xylem	1
H	Gallery	1
H	Surrounding xylem	1
N	Gallery	4.27
N	Surrounding xylem	1.28

Table S5: Carbon:phosphorus (C:P), C:nitrogen (N), N:P, N:sulfur (S), N:Ca (calcium), N:potassium (K), N:Mg (magnesium), N:Mn (manganese), and N:Zn (zinc) ratios (molar, as different elements were compared) for each of the three types of samples of *Trypodendron lineatum* and *Xyleborinus saxesenii*.

Trypodendron lineatum		*Xyleborinus saxesenii*	
C:P Control	29225.61	C:P Control	36061.25
C:P Gallery	8231.68	C:P Gallery	2210.59
C:P Surrounding xylem	28539.64	C:P Surrounding xylem	20066.83
C:N Control	3523.37	C:N Control	1766.57
C:N Gallery	1135.91	C:N Gallery	416.24
C:N Surrounding xylem	4412.99	C:N Surrounding xylem	1385.25
N:P Control	8.3	N:P Control	20.41
N:P Gallery	10.08	N:P Gallery	5.31
N:P Surrounding xylem	6.47	N:P Surrounding xylem	14.49
N:S Control	6.22	N:S Control	10.93
N:S Gallery	10.08	N:S Gallery	14.06
N:S Surrounding xylem	5.64	N:S Surrounding xylem	13.85
N:Ca Control	0.84	N:Ca Control	2.91
N:Ca Gallery	1.5	N:Ca Gallery	8.76
N:Ca Surrounding xylem	0.53	N:Ca Surrounding xylem	4.07
N:K Control	1.21	N:K Control	1.09
N:K Gallery	2.23	N:K Gallery	2.12
N:K Surrounding xylem	0.77	N:K Surrounding xylem	1.37
N:Mg Control	3.85	N:Mg Control	3.7
N:Mg Gallery	6.96	N:Mg Gallery	9.28
N:Mg Surrounding xylem	2.52	N:Mg Surrounding xylem	5.08
N:Mn Control	190.73	N:Mn Control	8.85

N:Mn Gallery	236.06	N:Mn Gallery	26.18
N:Mn Surrounding xylem	65.29	N:Mn Surrounding xylem	11.48
N:Zn Control	84.32	N:Zn Control	196.32
N:Zn Gallery	188.89	N:Zn Gallery	569.33
N:Zn Surrounding xylem	57.22	N:Zn Surrounding xylem	294.49

Chapter 5

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Working title: Volatiles and amino acids in bark and ambrosia beetle fungi

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Abstract

Bark and ambrosia beetles are associated with a broad range of fungi with several beneficial or competitive effects. They fulfill major steps of their life cycle either under the bark within the phloem (bark beetles, *sensu lato*) or the sapwood (ambrosia beetles, *sensu stricto*) of trees in almost complete darkness. Recent research showed that especially fungal volatile organic compounds (VOCs) have the potential to serve as key players for bark beetles to distinguish between beneficial and antagonistic fungi at short range within their tunnels. Here, we aimed to investigate a broad range of mostly understudied bark and ambrosia beetle fungi including free-living species and antagonists for their amino acid content and the derived volatile organic compounds. LC-MS/MS revealed striking differences between the amino acid composition of bark and ambrosia beetle fungi. GC-MS measurements on the other hand indicated a high diversity of emitted VOCs, where the availability of especially aliphatic and aromatic alcohols and esters, but also of several mono- and sesquiterpenes was much more dominant in bark and ambrosia beetle fungi compared to free-living species and antagonists. Our results highlight the importance of amino acids and VOCs within symbiotic bark and ambrosia beetle fungi and suggest their crucial role as essential drivers in maintaining the beetle-fungus symbioses.

Keywords: Mutualistic fungi, bark and ambrosia beetles, VOC, aliphatic esters/alcohols, aromatic esters/alcohols, terpenes, amino acids.

Introduction

Bark beetles (*sensu lato*) (Curculionidae: Scolytinae) are generally characterized by their ability to colonize several kinds of woody substrates such as the outer bark (e.g. *Dendroctonus brevicomis*), the phloem (e.g. *Ips typographus*), or the sapwood (e.g. *Xyleborinus saxesenii*) of almost every soft- and hardwood (e.g. 1). Most species spend almost their entire life in excavated galleries within woody tissue, in which they fulfill nearly all major steps of their life cycle. Several studies revealed their relationship with a broad range of microorganism like yeast, bacteria, and especially filamentous fungi, from which certain mutualistic species of the latest group are well known for their beneficial effects towards their beetle hosts (1-3). Indeed, filamentous fungi can have crucial functions within the beetle-fungus mutualism such as the detoxification of chemical tree-defense compounds (e.g. phenols) or the suppression of ubiquitous antagonistic fungi (4-6). However, certain highly evolved fungi can also serve as an efficient source of nutrition. This is especially the case in sapwood-colonizing ambrosia beetles (*sensu stricto*), which have long been known for their obligate mutualism with nutritional fungi. These fungal mutualists, which are transmitted vertically from the beetles in highly specialized and species-specific internal glands (mycetangia or mycangia) (such structures also evolved in some bark beetles) serve as the sole source of nutrition and are actively farmed within the beetles´ galleries (1, 7-10). Currently, a nutritional character of mutualistic fungi is believed for the majority of studied ambrosia beetles, although only very few studies including few or individual fungi focused on this issue (9, 11-15). The exact role is still unclear for the majority of regarded mutualists of ambrosia beetles and phloem-colonizing bark beetles. Mechanisms that maintain the relationship, apart from vertical transmission, of bark and ambrosia beetles with filamentous fungi are only poorly investigated and several questions remain open.

As both bark and ambrosia beetles and their mutualistic fungi are foraging in the dark, mechanisms must have evolved that allow beetles to recognize fungal associates. Partner choice in bark and ambrosia beetles could be due to the production of fungal volatile organic compounds (FVOCs), similar as described for the Drosophila-yeast system (see 16). The presence of FVOCs as well as possible functions in bark and ambrosia beetle systems has only been poorly investigated and only in a few species in the past years (17-18).

FVOCs can have the availability to be of crucial importance in maintaining beetle-fungus symbioses. Indeed, recent research showed that VOCs emitted by fungal associates of the European spruce bark beetle, *Ips typographus*, and other bark beetles (certain species in the genera *Dendroctonus*, *Ips*, *Pityogenes*) can influence the behavior, allowing beetles to distinguish between beneficial and non-beneficial/antagonistic fungi (see 17, 19). Here, several FVOCs could be identified and were classified into aliphatic/aromatic alcohols and esters as well as certain mono- and sesquiterpenes. Although the pleasant odor emitted by ambrosia beetle fungi has long been known (20) only few ambrosia beetle systems were examined since this time (21-25), while the majority of known ambrosia beetle fungi as well as the role of FVOCs in the ambrosia beetle-fungus mutualism remains unstudied. Only one study identified a possible attractive role of emitted FVOCs towards ambrosia beetle hosts (18), although the authors applied only behavioral assays with beetles and fungi and did not identify individual involved FVOCs.

Woody tissue, which serves as the breeding substrate for bark and ambrosia beetles, is generally an extremely poor source of many essential nutrients (26-27). For instance, elements like nitrogen, sulfur, and phosphorus are only available in very low amounts (especially within the sapwood), which indicates the challenging task for both beetles and fungi to survive and reproduce in this challenging environment (see

26, 28). Besides the low availability of certain elements, several free and bounded amino acids are present within wood, but their concentration is rather low (29-32). This is especially the case within the sapwood, where much lower concentrations of free amino acids are available compared to phloem (see 32). For the production of FVOCs (as well as in terms of their nutritional quality for the beetles), associated fungi are necessarily depending on the availability of nitrogen and/or some amino acids to synthesize especially the amino acids phenylalanine (Phe), leucine (Leu), and isoleucine (Ile), which are crucially needed to emit several aliphatic/aromatic alcohols and esters (see 17, 33). To date, adequate studies dealing with the amino acid content of bark and ambrosia beetle fungi are mostly absent (e.g. 34), but are necessary to gain novel insights into the nutritional character of symbiotic fungi as well as to understand their capabilities to produce certain FVOCs.

Several genera of bark and ambrosia beetles are known to have the potential to cause high economic damages in conifer and broadleaf forests and plantations around the world (e.g. *Dendroctonus*, *Ips*, *Euwallacea*, *Xyleborinus*, *Xylosandrus*) and can act as severe pests in such ecosystems (3, 35-39). Therefore, research on mechanisms that maintain the beetle-fungus mutualisms are important to understand the ecology of such systems and thus, to develop and implement possible management strategies.

Here, we examined 25 species from four convergently evolved clades of bark and ambrosia beetle associated fungal symbionts, including 16 mutualistic associates and five possibly beneficial symbionts for their ability to produce volatile organic compounds as well as for their total free amino acid content. Additionally, we included four common fungal antagonists as well as one free-living, closely related, non-mutualist filamentous fungus to test for possible phylogenetic effects. Moreover, the total of 30 examined fungi included nine species with unknown roles within the

investigated beetle-fungus systems. We aimed to (**i**) identify emitted volatile organic compounds within the most important clades of bark and ambrosia beetle fungi (Saccaromycetales, Ophiostomatales, Microascales, and Hypocreales) (see Fig. 1), and (**ii**) to compare our findings between all examined fungi based on two applied classification systems, where species were either grouped in terms of their respective host (ambrosia beetle, bark beetle, various insects/others) or regarding their role within the beetle-fungus symbiosis (mutualists, possibly beneficial symbionts, role unknown/antagonists). As the availability of amino acids is of crucial importance for fungi in terms of nutritional issues as well as their ability to emit certain volatile organic compounds, we aimed to (**iii**) identify free amino acids within biomass of all examined fungi and (**iv**) to compare our findings based on the two classification systems mentioned above.

Materials & Methods

Fungal species

Here, we examined 25 common fungal associates of several widespread bark and ambrosia beetle species from three ascomycete orders (Ophiostomatales (17 × Ophiostomataceae), Microascales (4 × Ceratocystidaceae, 1 × Microascaceae), and Hypocreales (2 × Nectriaceae)) as well as from one Saccharomycete order (Saccharomycetales (1 × Alloascoideaceae)). Further, we included four common fungal antagonists in the three ascomycete orders Hypocreales (1 × Cordycipitaceae), Sordariales (1 × Chaetomiaceae) and Eurotiales (2 × Aspergillaceae) as well as the free-living nematophagous fungus *Esteya vermicola*, serving as a non-beetle-associated phylogenetic control in the Ophiostomatales (Ophiostomataceae) (for an overview of all strains see Table 1 & Suppl. Table S1).

All fungal isolates are maintained at -80°C in glycerol (80%) and glycerol / peptone (80% / 1%, Roth; Germany) at the Department of Forest Entomology and Protection (University of Freiburg, Germany). Origins of each fungal strain are given in Suppl. Table S1. Prior to the experiment, all fungi were revived on 4% potato dextrose agar (PDA; Sigma Aldrich, Germany) serving as our experimental culture medium, and maintained at 4°C until they were subcultured in fresh PDA at room temperature (RT) for 4-6 days before experimental use.

All fungal species were included within a phylogenetic replacement to visualize (**i**) the independently evolved phylogenetic lineages of all examined species and (**ii**) to indicate each classification (by host and by role) as well as each sub-classification (see statistical analyses).

DNA extraction and LSU / ITS / βT barcoding

DNA extraction and barcoding were conducted at the Department of Animal Ecology and Tropical Biology (University of Würzburg, Germany). Here, fungal samples were first homogenized by grinding in liquid nitrogen. DNA was extracted afterwards using the NORGEN Biotek Corp. fungi/yeast genomic DNA isolation kit. Primarily, we used the large subunit (LSU) of the ribosomal RNA gene for the identification and phylogenetic placement of isolates (Fig. 1, Suppl. Table S1). For amplification, we used the common primers LROR-F (GTACCCGCTGAACTTAAGC) and LR5-R (ATCCTGAGGGAAACTTCG) (40-41). As annealing temperature, we applied 55.5°C. The resulting sequence lengths were about 830 to 880 bp.

As few samples repeatedly failed to amplify using the LSU primers, we either amplified the internal transcribed spacer (ITS) of the ribosomal RNA or the beta-tubulin gene (βT) to confirm the specimen's identity. The ITS region was amplified following the protocol in (42). For amplifying βT, we used the primers T10-F (ACGATAGGTTCACCTCCAGAC) and Bt2b-R

(ACCCTCAGTGTAGTGACCCTTGGC) (43-44) and 57.5°C as annealing temperature. Sequence lengths contained around 300 to 400 bp.

We used a similar master-mix for all PCR reactions for barcoding the LSU, ITS and βT region ((PCR Master-mix for 50 µl: 25 µl 2x phusion high-fidelity PCR master mix with GC buffer (Thermo Scientific™, Germany), 2.5 µl forward primer 1 (10 µM, Eurofins Genomics, Germany), 2.5 µl reverse primer 2 (10 µM, Eurofins Genomics, Germany), 18 µl ddH20, 2 µl template (usually 1:10 diluted). We applied the following PCR conditions to all three genetic markers: 98°C for 30 s, followed by 35 cycles at 98°C for 10 s, 55.5°C, 54.5°C or 57.5°C (for LSU, ITS and βT, respectively) for 30 s and 72°C for 20s, ending with 72°C for 10 min including a storage temperature of 5°C.

For purifying extracted DNA, we used the Wizard® SV gel and PCR clean-up system (Promega, Germany) after performing gel electrophoresis. Sequencing was accomplished by Eurofins (Eurofins Genomics, Germany). Each sequence quality was verified using the "SnapGene® Viewer" 3.2 (SnapGene software, from GSL Biotech; available at snapgene.com). If necessary, automatically deduced sequences were manually corrected. We finally identified fungal species using BLASTn NCBI (45). All obtained sequences were uploaded to the NCBI GenBank database. Accession numbers were added to Suppl. Table S1.

Phylogenetic replacement

We used 23 of our sequences of the large subunit (LSU) for the maximum likelihood analyses and construction of a phylogenetic tree (see Suppl. Tables S1 - S2). The remaining seven sequences of examined fungi as well as the 51 fungal outgroup sequences were received from the NCBI GenBank database (see Suppl. Table S2). Individual sequences were prepared by using GeneDoc 2.7 (46) and MEGA 7.0 (47) and were additionally compared with the received output from Clustal Omega (48).

We used the IQ-Tree-Web-Server (49-50) for our phylogenetic analyses utilizing the ultrafast bootstrap analysis (51). According to the web server FindModel (52), available at hiv.lanl.gov/content/sequence/findmodel/findmodel.html), we chose the generalized time reversible model (GTR) plus gamma (+G) with rate heterogeneity (rate categories – 4) and combined with 10.000 bootstrap alignments as our substitution model. FigTree was used for visualizing our phylogenetic tree ((http://tree.bio.ed.ac.uk/software/figtree).

We did not include the findings from our phylogenetic analysis within the results, as the relationships among the studied fungi are already known (53-56). Here, we aimed to give an overview over our choice of fungi and the different examined clades as well as to visualize the classification of our species in an appealing way (see Fig. 1).

Semi-quantitative analysis of fungal volatile organic compounds over culture development

The collection and analyses of volatile organic compounds (hereafter termed VOC) were conducted at the Max-Planck Institute for Chemical Ecology (Jena, Germany), where we collected VOCs at two different time intervals (6 and 12 days after culture inoculation, at room temperature [RT]). Polydimethylsiloxane (PDMS: (57)) sorbents were utilized to collect VOCs from tested fungi (N = 30; 4 - 5 replicates per fungus, see Suppl. Table S3) inoculated on PDA. Further, we included a control (N = 5, pure PDA) for both examined time points. VOCs identified from our control samples were excluded from the remaining samples and were not part of the analyses. For the collection of VOCs, we embedded a sterile metal wire equipped with two till three PDMS tubes (~5mm) per plate in the PDA (within a UV-sterilized working bench using oven-sterilized metal forceps). All plates were sealed with parafilm and headspace VOCs were collected for 30 min. Afterwards, each PDMS tube was

carefully removed from the metal wires and transferred to a 1.5 mL brown glass vial and stored at -20°C until further processing.

For analysis, we used a GC-2010 plus gas chromatograph, which was coupled to a MS-QP2010 quadrupole mass spectrometer with a TD-20 thermal desorption unit (Shimadzu, Japan) and a GC Cryo-Trap (Tenax®). Applied settings as well as the separation and detection followed the method described in (19) (analytes were injected onto a Rtx-5MS GC column using split mode [1:100]).

Images of all examined plates were taken (Sony alpha 5000, 12 × in macro mode) subsequently after volatile collection for each investigated time interval (including all replicates) and analyzed for surface area using ImageJ software (version 1.52a). In aim to obtain semi-quantitative results (counts/mm2) for further statistical analysis, we divided the output of the VOC analysis (peak area in counts) through the calculated surface area (mm2).

Quantitative analysis of fungal amino acids over culture development

Studied species (N = 30; 4 – 5 replicates per fungus, see Suppl. Table S3) were cultured on PDA (at the Max-Planck Institute for Chemical Ecology) supplemented with a piece of sterilized cellophane (6 × 6 cm, NeoLab, Germany), which was added to each petri dish immediately after the media had cooled down. Afterwards, we inoculated a plug of pre-cultured fungal mycelium (containing PDA; Ø 3 mm, using a cork borer) to the center of each plate and incubated them tightly sealed with parafilm at RT. At the two examined time intervals (6 and 12 days), we removed pure fungal mycelium from each plate and transferred it into pre-weighted Eppendorf tubes (1.5mL; Eppendorf, Germany). After determining the weight of each individual sample (5 – 100 mg per sample), we added 300µl Methanol (ultrapure, for liquid chromatography; Merck, Germany) as extraction solvent and homogenized samples

using a beat beater (SKANDEX S-7) for 3 min at moderate intensity. Afterwards, samples were centrifuged (10000 rpm for 5 min) and pure supernatant was removed.

We quantified the following 18 free amino acids within all examined fungi: alanine (Ala), serine (Ser), proline (Pro), valine (Val), threonine (Thr), isoleucine (Ile), leucine (Leu), aspartic acid (Asp), glutamic acid (Glu), methionine (Met), histidine (His), phenylalanine (Phe), arginine (Arg), tyrosine (Tyr), tryptophan, (Tyr), asparagine (Asn), glutamine (Gln), and lysine (Lys). Here, amino acids were quantified by LC-MS/MS on a Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8 µm, Agilent Technologies, Germany) using a TripleQuad-MS system (Sciex QTRAP6500), after diluting the extract (1:50) with water containing 10 µg ml-1 of a mix of 15N/13C labelled amino acids (Isotec, Miamisburg, OH, USA) (for details on method see (58)). We quantified amino acids (in nmol/mg = µmol/g) relative to the peak area of their corresponding labelled amino acids, except for tryptophan (relative to phenylalanine, response factor 0.42). Remaining culture supernatant was stored at -20°C.

Statistical analyses

Data classification - Fungi

Prior the statistical analyses, we classified all examined fungal species into two groups, indicating (**i**) the associated beetle group (classification by host) as well as (**ii**) the role of each fungus (classification by role) within the beetle-fungus symbiosis. Here, each of the two classification systems was subdivided into three further groups (based on references in Table 1 and personal communication), serving as the base for further analyses. For the classification by host (**i**), species were added more generally to the appropriate group of (a) ambrosia beetles (including mutualists and possibly beneficial symbionts; N = 10), (b) bark beetles (including mutualists and possibly beneficial symbionts; N = 11) and (c) various insects/free-living species (e.g.

role unknown, antagonists, others; N = 9). Within the classification by role (**ii**), we grouped our fungi more specifically into (a) mutualists (N = 16), (b) possibly beneficial symbionts (N = 5) and (c) others (e.g. Role unknown, antagonists; N = 9) regardless of their association with a bark or an ambrosia beetle (see Table 1).

Data classification – Fungal VOCs

Further, all identified VOCs collected at day 6 and day 12 were classified into nine chemical groups: Aliphatic esters, aromatic esters, aliphatic alcohols, aromatic alcohols, aliphatic ketones, aromatics, monoterpenes, sesquiterpenes, and others. For this purpose, we generated a second dataset (Grouped VOCs), based on the means of all replicates per fungus and individual VOC from the original dataset. Here, means of each individual VOC were summed up based on the appropriate chemical group. As we automatically loose some information when classifying all identified VOCs into the different chemical groups, we decided to generate a third dataset (Number of compounds), containing the absolute number of VOCs, which were produced by each fungus within each classified chemical group.

Statistics

All statistical analyses were conducted in R (version 1.2.5033). Here, we aimed to test for statistical differences regarding the amino acid and VOC content (the latest divided into grouped VOCs and number of compounds; see above) between studied fungi based on the two applied classification systems (by host and by role). Therefore, we applied several 3-dimensional non-metric multidimensional scaling (NMDS) analyses using the packages “vegan” (59) and “vegan3D” (60), followed by an adonis and pairwise permanova (number of permutations = 999) using the package “RVAideMemoire” (61) to identify differences among the two classifications. Additionally, we visualized the abundance of each (**i**) individual VOC within the eight

(as we did not visualize the group others) chosen chemical groups (based on the mean) and (**ii**) the respective absolute number of volatiles produced within this group as well as (iii) the absolute amino acid content for each individual fungus (based on the mean) using heatmaps in ggplot, where we applied the following packages: “plyr” (62), “dplyr” (63), “pheatmap” (64), “viridis” (65), “RColorBrewer” (66), “gplots” (67), and “reshape2” (68).

Further, we generated individual heatmaps for the three amino acids phenylalanine (Phe), isoleucine (Ile) and leucine (Leu) applying the identical packages as described above. These three amino acids are well known for their importance regarding the availability of especially aliphatic and aromatic alcohols and esters as the majority of VOCs within these groups derived from Phe, Leu, and Ile (33), (17), (69). To test for statistical differences between the content of these three amino acids within the studied fungi (based on the two applied classification systems), we used a one-way anova followed by a tukey HSD multiple comparison test.

Results

Semi-quantitative analysis of fungal VOCs over culture development

In total, we identified 81 different VOCs from all examined fungi (N = 30 species) after an incubation time of 6 days, while 76 different VOCs were detected after 12 days. In general, all compounds were classified into the following eight chemical groups (number of VOCs at day 6 and 12 in brackets): aromatic alcohols (day 6: N = 3, day 12: N = 3), aromatic esters (day 6: N = 4, day 12: N = 3), aliphatic alcohols (day 6: N = 6, day 12: N = 7), aliphatic esters (day 6: N = 24, day 12: N = 18), aliphatic ketones (day 6: N = 3, day 12: N = 3), aromatics (day 6: N = 2, day 12: N = 2), monoterpenes (day 6: N = 10, day 12: N = 10), and sesquiterpenes (day 6: N = 23, day 12: N = 23).

According to the number of VOCs based on the classification by host, the NMDS analysis and following posthoc tests revealed a significant higher number of VOCs (after an incubation time of 6 days, stress level = 0.089) produced by ambrosia beetle fungi ($p = 0.003$) and bark beetle fungi ($p = 0.012$) relative to “others” (including free-living species and species associated with various insects). We observed a trend between ambrosia beetle fungi and bark beetle fungi ($p = 0.06$), although there was no significant difference. Within the classification by role, mutualistic fungi ($p = 0.0075$) as well as possibly beneficial symbionts ($p = 0.0075$) highly differ from “others”, while there is no difference between mutualists and possibly beneficial symbionts ($p = 0.32$). After an incubation time of 12 days (stress level = 0.086), our analysis revealed that ambrosia beetle fungi ($p = 0.006$) as well as mutualists ($p = 0.027$) still differ regarding the amount of produced VOCs from “others” (see Supplementary Figure S1a & b). The generated heatmap indicates a higher abundance of especially aliphatic alcohols and aliphatic esters produced by studied ambrosia and bark beetle fungi as well as by mutualists and possibly beneficial symbionts towards the group others (see Fig. 2 a & b). Further, we detected the highest number of monoterpenes and sesquiterpenes within the mutualistic ambrosia beetle fungi *Alloascoidea hylecoeti* (8 monoterpenes, see also day 6: Supplementary Fig. S10 a & b; day 12: Supplementary Figure S11 a & b) and *Graphium euwallaceae* (14 sesquiterpenes, see also day 6: Supplementary Fig. S12 a & b; day 12: Supplementary Fig. 13 a & b).

The NMDS analysis and following posthoc tests of VOCs, which were classified into eight chemical groups (based on summed up values of individual volatiles within each chemical group; see above) indicates slight differences within the classification by host. Here, ambrosia beetle fungi ($p = 0.048$) and bark beetle fungi ($p = 0.048$) slightly differ towards “others” after an incubation time of 6 days

(stress level = 0.087). The classification by role revealed no differences between the three groups of studied fungi. After 12 days (stress level = 0.087), ambrosia beetle fungi ($p = 0.03$) and bark beetle fungi ($p = 0.03$) still slightly differ from the group others, while no differences were found between groups within the classification by role. The generated heatmaps indicate that within the group aromatic alcohols especially phenylethyl alcohol was the major compound identified within ambrosia and bark beetle fungi as well as within mutualists and possibly beneficial symbionts (day 6: Fig. 3 a & b; day 12: Supplementary Fig. S2 a & b).

We detected phenethyl acetate (aromatic esters) as the main compound within ambrosia and bark beetle associated fungi, while it was especially dominant within mutualistic fungi (day 6: Fig. 4 a & b; day 12: Supplementary Fig. S3 a & b). Here, species from the group “others” did not produce any aromatic esters. Ethanol and 1-Butanol-3-methyl (aliphatic alcohols) were dominantly produced by ambrosia and bark beetle fungi as well as mutualistic fungi and possibly beneficial symbionts and only few studied species from the group “others” also produced these compounds. Further, we found isobutyl alcohol to be almost exclusively produced by bark beetle fungi (day 6: Fig. 5 a & b; day 12: Supplementary Fig. S4 a & b).

VOCs within the aliphatic esters were generally most dominant within studied fungi. Here, especially ethyl acetate and isoamyl acetate were the major compounds produced by ambrosia and bark beetle fungi as well as mutualists and possibly beneficial symbionts (day 6: Fig. 6 [Classification by host] and Fig. 7 [Classification by role]; day 12: Supplementary Fig. S5 a & b). In general, mutualistic fungi showed the highest diversity of aliphatic esters. Further, we detected three VOCs within the aliphatic ketones (day 6: Supplementary Fig. S6; day 12: Supplementary Fig. S7), which were produced by three fungi (mutualistic ambrosia beetle fungus: *R. sulphurea*; Others: *Chaetomium globosum* and *Ophiostoma minus*) in low amounts.

Two VOCs within the aromatics (day 6: Supplementary Fig. S8; day 12: Supplementary Fig. S9) could be identified. Here, the mutualistic ambrosia beetle fungi *Graphium euwallaceae* was the only fungus that produced high amounts of styrene, while benzaldehyde was solely produced in low amounts by few bark beetle fungi. Species from the "group" others did not produce any aromatics.

Ten monoterpenes could be identified within all examined species, while the majority of compounds were produced by *A. hylecoeti* (see above). Only for three further fungal species, we could identify one monoterpene for each species which was produced in rather low amounts (day 6: Supplementary Fig. 10 a & b; day 12: Supplementary Fig. 11 a & b). Besides *G. euwallaceae*, which produced the highest number of different sesquiterpenes (see above), few other species (mutualistic ambrosia beetle fungi: *R. sulphurea* and *R. montetyi*; possibly beneficial bark beetle symbionts: E. polonica, *G. penicillata*, and *L. abietinum*) produced lower amounts of certain sesquiterpenes (day 6: Supplementary Fig. 12 a & b; day 12: Supplementary Fig. 13 a & b). For the majority of individual produced VOCs within all chemical groups, the abundance was highest after an incubation time of 6 days, while it decreased for most volatile compounds after an incubation time of 12 days.

Quantitative analysis of fungal amino acids over culture development

The NMDS analysis and following posthoc tests (including all quantified amino acids) revealed high differences in the amino acid composition (after an incubation time of 6 days [stress level = 0.07]) of ambrosia beetle fungi relative to bark beetle fungi ($p = 0.0015$) as well as of bark beetle fungi relative to "others" ($p = 0.0015$). Based on the classification by role, mutualistic fungi differ from possibly beneficial symbionts ($p = 0.05$), while possibly beneficial symbionts differ from the group others ($p = 0.012$). After an incubation time of 12 days (stress level = 0.097), our analysis revealed slight differences between ambrosia beetle fungi and bark beetle fungi ($p = 0.01$), as well

as between bark beetle fungi and the group others (p = 0.01). No differences were detected within the classification by role at day 12.

For phenylalanine, leucine, and isoleucine the Anova and Tukey HSD test revealed differences after an incubation time of 6 days within the classification by role, where possibly beneficial symbionts differed from “others” (p = 0.0014) as well as from mutualistic fungi (p = 0.01). We could not detect significant differences within the classification by host [but almost for ambrosia beetle fungi and the group others, p = 0.06] (see Fig. 8 a & b) as well as for both classifications after an incubation time of 12 days (Supplementary Fig. S14 a & b). The heatmaps visualizing all 18 quantified amino acids after an incubation time of 6 and 12 days were added to the supplementary material (see Supplementary Fig. S15 – S18).

Discussion

As bark and especially ambrosia beetles are excavating and colonizing their breeding systems in more or less complete darkness, they are expected to use FVOCs to distinguish among beneficial and “weedy” antagonistic fungi (i.e., partner choice). Here, we show that 11 independently evolved clades out of seven fungal orders associated with bark and ambrosia beetles (see Fig. 1) are emitting a much higher absolute number of different FVOCs compared to free-living and/or antagonistic fungi (Fig. 2 a). This pattern is even more visible, when we classified studied bark and ambrosia beetle fungi into mutualistic associates and possibly beneficial symbionts (Fig. 2 b). Although the analysis of FVOCs (summed up values classified into the eight chemical groups) revealed only a slight difference of bark and ambrosia beetle fungi (p = 0.03) relative to free-living and/or antagonistic fungi, the generated heatmaps are visualizing the generally higher abundances of particularly aliphatic and aromatic alcohols and esters within bark and ambrosia beetle fungi as well as within mutualistic associates and possibly beneficial symbiont (see Fig. 3 – 7).

Additionally, we found that amino acid content of bark beetle fungi highly differs relative to ambrosia beetle fungi as well as towards free-living and/or antagonistic fungi. Here, possibly beneficial symbionts differ in their total amino acid content relative to mutualistic associates and free-living and/or antagonistic fungi (see Suppl. Fig. 15 – 18). The amino acids phenylalanine, leucine, and isoleucine, which are precursors for the production of several aliphatic and aromatic alcohols and esters (e.g. (33, 69), were most dominant within possibly beneficial symbionts, followed by mutualistic associates, and free-living and/or antagonistic fungi with the lowest amounts (Fig. 8, Suppl. Fig. 14).

In contrast to ambrosia beetle fungi, where specific fungi are known to serve as obligate nutritional mutualists, the exact role of fungi associated with bark beetles is still not entirely clear (see (39, 70). Several species were classified as facultative mutualists in previous studies, while for others possible beneficial roles have been hypothesized (see Table 1 and respective literature). Interestingly, several examined bark and ambrosia beetle fungi as well as mutualists and possibly beneficial symbionts were producing similar FVOCs (especially in case of aromatic alcohols and aliphatic alcohols/esters) in moderate amounts (see Fig. 3 - 7). So far, some fungal associates of bark and ambrosia beetles have been shown to produce a broad range of FVOCs, mainly consisting out of aliphatic and aromatic alcohols and esters as well as out of few mono- and sesquiterpenes (e.g. 17, 19, 21-22, from which some can have attractive or repellent effects on the beetles (e.g. 18-19, 71-72). The attractive potential of certain ambrosia beetle fungi to their beetle hosts was already described for the ambrosia beetle genus *Xyleborus* as well as *Xylosandrus* (18, 21). Similar to the findings for the bark beetle *Ips typographus* (17, 19), beetles were highly attracted from specific fungi, while other fungi had a repellent effect. This behavior towards associated fungi indicates the prominent role of certain emitted

FVOCs from mutualistic fungi in ambrosia beetles and also bark beetle fungus associations. Specific FVOCs or blends may lead the beetles to areas within the colonized xylem of high nutritional value or indicate the presence/dominance of antagonistic fungi which needs to be removed to ensure the survival of the food fungus. As several bark and ambrosia beetles as well as their associated fungi are phylogenetically closely related (see Fig. 1) beneficial effects of several examined bark beetle fungi similar to associates of ambrosia beetles may be possible.

The presents and proportions of individual FVOCs highly differed among examined fungi. However, the most dominant aromatic alcohol emitted from especially bark beetle fungi as well as from 5 out of 10 ambrosia beetle fungi was phenylethyl alcohol (Fig. 3), while it was only found in two antagonistic or free-living species (*Ophiostoma minus* and *O. quercus*). Based on the classification by role, the majority of mutualistic fungi as well as all possibly beneficial symbionts emitted this compound. Besides our tested fungi, phenylethyl alcohol is also emitted from the yeast *Candida albicans* (see 73) as well as from the epiphytic fungus *Aureobasidium pullulans* (from which volatiles play a role as semiochemicals within eusocial wasps e.g. 74). Interestingly, this compound is also known for its antifungal effects towards certain pathogenic fungi (e.g. *Aspergillus flavus*, see 75-76). Indeed, *A. flavus* is a common antagonist within different ambrosia beetle systems (e.g. 77). Within the aliphatic alcohols, ethanol and 1-butanol-3-methyl were emitted from the majority of examined fungi (few fungi from the group others did not emit them), although species associated with bark and ambrosia beetles generally emitted higher amounts (Fig. 5). 1-Butanol-3-methyl, which is a natural product in yeast and fungi fermentation, is known to have antimicrobial properties towards pathogenic fungi (78- 79), what may be a hint for its use within the studied bark and ambrosia beetle fungi. Moreover, ethanol was emitted in much higher amounts from bark beetle fungi compared to

associates of ambrosia beetles. Ambrosia beetle fungi are known to produce, detoxify, and metabolize ethanol, while some previously tested bark beetle fungi are apparently not able to survive on ethanol enriched medium (see 5-6). Indeed, in (6), three common bark beetle fungi (*Endoconidiophora polonica*, *Grosmannia penicillata*, and *Ophiostoma bicolor*) were tested towards their ethanol tolerance/sensitivity and all fungi were highly inhibited through ethanol. Here, *E. polonica* and *O. bicolor* emitted either no or very low ethanol concentrations, while *G. penicillata* emitted the highest amounts of ethanol within all examined fungi. To confirm an intolerance of ethanol within bark beetle fungi, a broader range of species needs to be tested according to (6).

The majority of aliphatic esters (N = 24) were produced by certain bark and ambrosia beetle fungi, while only four aliphatic esters were produced by antagonistic and/or free-living fungi. Especially the yeast-like fungus *Alloascoidea hylecoeti*, *Ambrosiella hartigii* and *A. roeperi* as well as *Endoconidiophora polonica* were responsible for the high diversity of emitted compounds (see Fig. 6 – 7). Certain aliphatic esters such as ethyl acetate, isoamyl acetate, and ethylphenyl acetate are known to act as attractants within *Drosophila* (80), but their functions within the majority of bark and ambrosia beetles is mostly unclear (except *Ips typographus* see 19, where aliphatic esters are attractants to the beetles). Interestingly, the mutualistic *Dendroctonus brevicomis*-associated fungus *Ceratocystiopsis brevicomi*, emitted only ethyl acetate within the aliphatic esters in low amounts. Also the mutualistic *C. ranaculosus*, associated with *D. frontalis* emitted only few volatiles in low amounts. Possibly these beetles, which are colonizing the outer bark of infested trees (after brief feeding within the phloem as first stage larvae), where they excavate very short galleries (81-82), are not necessarily depending on fungal volatiles or other associated fungi (e.g. *Entomocorticium*) may overtake such effects.

Only few species emitted low amounts of monoterpenes (N = 4), while the mutualistic yeast-like ambrosia beetle fungus *Alloascoidea hylecoeti*, associated with the ship-timber beetle *Elateroides hylecoetus*, emitted 8 monoterpenes such as citronellol, citronellyl acetate, nerol, and geranyl acetate (Suppl. Fig. S10 – 11). As monoterpenes are generally known for their antifungal effects on several fungi (see (83-84), the high number and amounts produced by *A. hylecoeti* is quite surprising and to our knowledge unique within a bark or ambrosia beetle system. Indeed, the symbiosis with a fungus that emits several monoterpenes would fit well with the ecology of *E. dermestoides*, whose larvae bore solitary tunnels in which they farm their symbiont (85-86). As only one larva is present within each tunnel, there is a high risk that either the larva or the fungus gets overgrown by ubiquitous antagonistic fungi. Here, emitted monoterpenes with high antifungal capabilities may be beneficial in terms of maintaining the symbiosis, as antagonistic fungi may be suppressed. To prove this, bioassays with *A. hylecoeti* towards antagonistic fungi as well as antagonistic fungi towards individual monoterpenes are needed. However, sesquiterpenes are even known for several functions like having antimicrobial properties, acting as insect repellents or attractants and can even have synergistic effects to other microbial organisms (87 and references therein). Within our study, the mutualistic ambrosia beetle fungus *Graphium euwallaceae* associated with the polyphagous shot hole borer *Euwallacea fornicates* was the only fungus that produced several sesquiterpenes (N = 14) such as aristolene, valencene, alpha-selinene, beta-Gurjunene as well as several unknown further sesquiterpenes (Suppl. Fig. S12 – 13). This indicates a crucial but unknown role of *G. euwallaceae* within the beetle-fungus symbiosis and makes further studies necessary.

The presents of certain amino acids (especially Phe, Ile, and Leu) highly influence the availability of fungi to emit volatile organic compounds, which can act as

attractants (19, 33, 69). Further, the total amount of free and/or bonded amino acids may give hints about the nutritional quality of fungi depending on which specific amino acids are available and in what proportions (see 88 and references therein), although especially the combination of amino acids, vitamins, fatty acids, and sterols are mainly responsible for the nutritional character of beetle-associated fungi. Especially mutualistic fungi of ambrosia beetles are thought to have a high nutritional value, as they are serving as the only food source for their beetle hosts (e.g. 8, 14). Here, the identification of involved free amino acids within fungal biomass revealed striking differences between ambrosia beetle fungi and bark beetle fungi as well as between mutualistic associates and possibly beneficial symbiont (Suppl. Fig. S15 – S18). All examined fungi (including the group others) contained 18 free amino acids but differ regarding the proportions of individual amino acids. Here, it is important to compare the proportions of different amino acids between all examined fungi, what makes further analyses necessary. This will allow us to determine which individual amino acids may play a role in terms of nutrition. Phenylalanine, isoleucine and leucine are apparently enriched within bark and ambrosia beetle fungi, although we could not reveal significant differences. Here, possibly beneficial symbionts showed the highest amount of these three amino acids followed by mutualistic associates. These findings fit well with emitted FVOCs, as especially the FVOCs-rich species, such as *A. hylecoeti*, *Ambrosiella* spp., *E. polonica*, *G. penicillata* as well as *O. bicolor* were highly enriched with phenylalanine, isoleucine, and leucine (see Fig. 8). This indicates that FVOC may be honest signals for nutritional quality of fungi in terms of amino acid content. However, the content of amino acids within tissue of examined fungi should be discussed with appropriate care, as fungi were cultivated on an artificial amnio acid rich medium although clear differences between classified groups as well as between individual species were found.

Based on (19), it would be interesting for further studies to examine the exact role of certain FVOCs within several ambrosia and bark beetle systems and to prove that the majority of beetle hosts are attracted by FVOCs and well as by artificial blends of their associated fungi. Here, behavioral assays with beetles including associated beneficial and antagonistic fungi as well as with artificial blends and individual volatile compounds may help to understand underlying processes. Additionally, it would be interesting to test possible antimicrobial effects of individual volatiles emitted by mutualists towards antagonists to clarify which specific compounds are able to inhibit antagonistic fungi and how species-specific this may be. Moreover, the nutritional quality of fungi associated with ambrosia and bark beetles is only poorly investigated. To date, studies investigating the content of vitamins, amino acids, fatty acids and sterols within bark and especially ambrosia beetle fungi are only poorly available (except few studies investigating individual species, e.g. 13-14, 89- 90). Such studies are crucially needed to answer long-standing questions about the actual nutritional quality of such beetle-associated fungi and how this quality related to the FVOCs emitted.

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Competing Interests:

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1: Phylogenetic placement based on 81 fungal LSU sequences. Colored (red, green, and blue) species were examined within this study (N = 30), while un-colored (black) species act as a fungal outgroup (N = 51). The LSU region from 23 of 30 examined fungi was sequenced by us and utilized for the maximum-likelihood analysis. The remaining seven sequences of *Raffaelea sulphurea* (P159), *Raffaelea montetyi* (P105), *Raffaelea amase* (P45), *Estya vermicola* (P167), *Raffaelea canadensis* (P23), *Leptographium longiclavatum* (D1), and *Ceratocystiopsis ranaculosus* (P320) (strain IDs in brackets) as well as of the 51 fungal outgroup sequences were obtained from NCBI GenBank. For accession numbers and further information to each strain see Supplementary Tables S1 – S2. We indicated the order of each species within the phylogenetic placement and subdivided the studied Ophiostomatales and Microascales into their four and two sub-clades, respectively. Two classification systems were applied to all examined fungi based on (i) the associated host (ambrosia beetle, bark beetle, and other/various insects) as well as on (ii) the role of each fungus (mutualist, possibly beneficial symbiont, and role unknown/antagonists).

Fig. 2: Heatmap visualizing the absolute number of identified volatiles (N = 81) per examined fungus (N = 30) after a cultivation time of 6 days, which were classified into nine different chemical groups (including the group “other volatiles”). Each number represents the absolute number of volatiles produced by one fungus within each chemical group. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]). We indicated

significant differences within each classification system by adding p-values to the legend of a and b, which we obtained from a NMDS analysis.

Fig. 3: Heatmap visualizing the concentration of each volatile (N = 3) classified into the group of aromatic alcohols per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. 4: Heatmap visualizing the concentration of each volatile (N = 4) classified into the group of aromatic esters per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. 5: Heatmap visualizing the concentration of each volatile (N = 6) classified into the group of aliphatic alcohols per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications

"various insects" and "free-living"]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications "role unknown" and "antagonists"]).

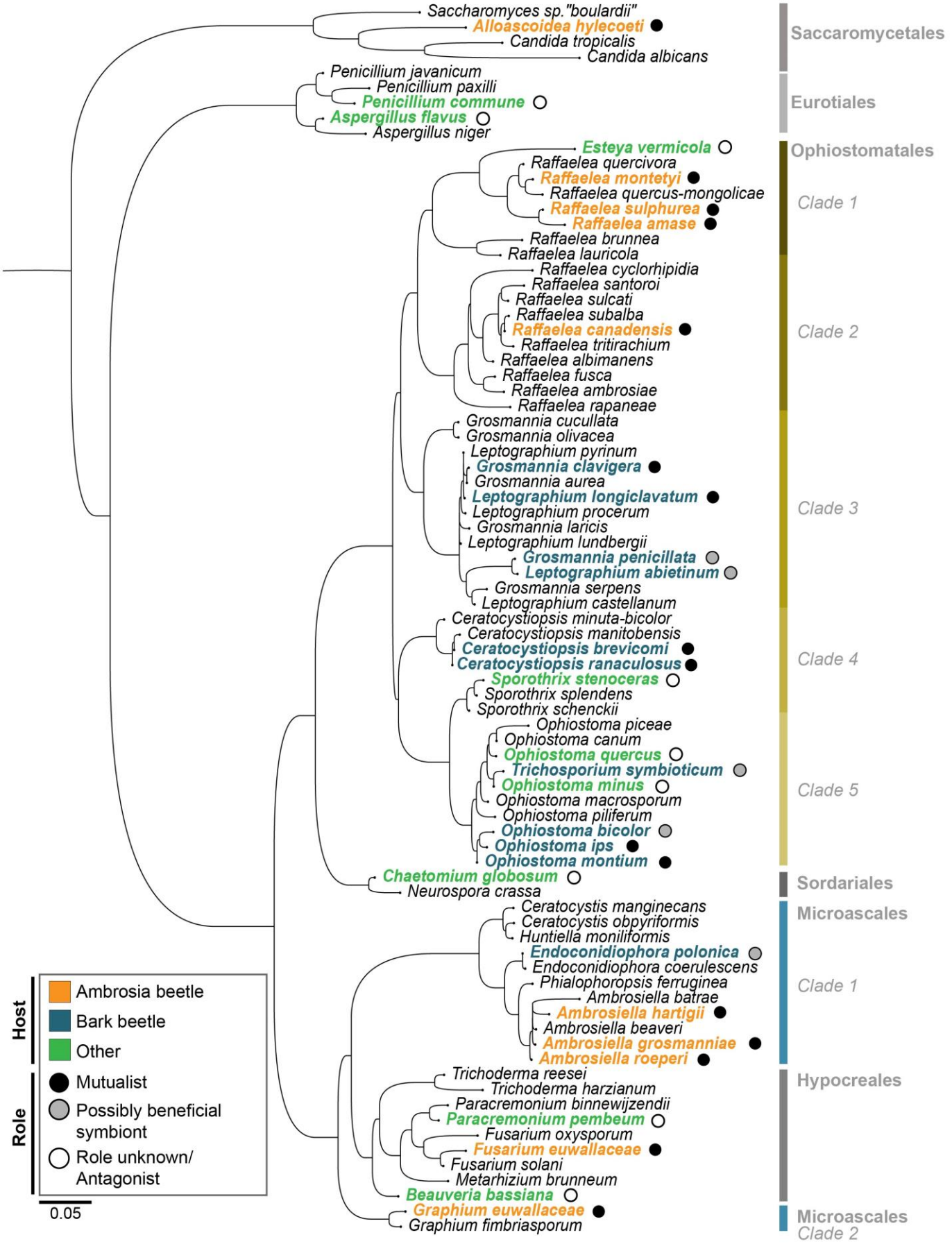
Fig. 6: Heatmap visualizing the concentration of each volatile (N = 24) classified into the group of aliphatic esters per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Here, the classification system based on the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications "various insects" and "free-living"]) is shown.

Fig. 7: Heatmap visualizing the concentration of each volatile (N = 24) classified into the group of aliphatic esters per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Here, the classification system based on the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications "role unknown" and "antagonists"]) is shown.

Fig. 8: Heatmap visualizing the absolute concentration of the three amino acids phenylalanine (Phe), leucine (Leu), and isoleucine (Ile) within each examined fungus (N = 30) after a cultivation time of 6 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications "various insects" and "free-living"]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications "role unknown" and "antagonists"]). We

indicated significant differences within each classification system by adding p-values to the legend of a and b, which we obtained from a one-way ANOVA analysis.

Fig. 1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Candida albicans
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Penicillium paxilli
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Aspergillus flavus
Aspergillus niger
Esteya vermicola
Raffaelea quercivora
Raffaelea montetyi
Raffaelea quercus-mongolicae
Raffaelea sulphurea
Raffaelea amase
Raffaelea brunnea
Raffaelea lauricola
Raffaelea cyclorhipidia
Raffaelea santoroi
Raffaelea sulcati
Raffaelea subalba
Raffaelea canadensis
Raffaelea tritirachium
Raffaelea albimanens
Raffaelea fusca
Raffaelea ambrosiae
Raffaelea rapaneae
Grosmannia cucullata
Grosmannia olivacea
Leptographium pyrinum
Grosmannia clavigera
Grosmannia aurea
Leptographium longiclavatum
Leptographium procerum
Grosmannia laricis
Leptographium lundbergii
Grosmannia penicillata
Leptographium abietinum
Grosmannia serpens
Leptographium castellanum
Ceratocystiopsis minuta-bicolor
Ceratocystiopsis manitobensis
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Sporothrix stenoceras
Sporothrix splendens
Sporothrix schenckii
Ophiostoma piceae
Ophiostoma canum
Ophiostoma quercus
Trichosporium symbioticum
Ophiostoma minus
Ophiostoma macrosporum
Ophiostoma piliferum
Ophiostoma bicolor
Ophiostoma ips
Ophiostoma montium
Chaetomium globosum
Neurospora crassa
Ceratocystis manginecans
Ceratocystis obpyriformis
Huntiella moniliformis
Endoconidiophora polonica
Endoconidiophora coerulescens
Phialophoropsis ferruginea
Ambrosiella batrae
Ambrosiella hartigii
Ambrosiella beaveri
Ambrosiella grosmanniae
Ambrosiella roeperi
Trichoderma reesei
Trichoderma harzianum
Paracremonium binnewijzendii
Paracremonium pembeum
Fusarium oxysporum
Fusarium euwallaceae
Fusarium solani
Metarhizium brunneum
Beauveria bassiana
Graphium euwallaceae
Graphium fimbriasporum
Saccharomycetales
Eurotiales
Ophiostomatales
Clade 1
Clade 2
Clade 3
Clade 4
Clade 5
Sordariales
Microascales
Clade 1
Hypocreales
Microascales
Clade 2
Host
Ambrosia beetle
Bark beetle
Other
Role
Mutualist
Possibly beneficial symbiont
Role unknown/ Antagonist
0.05

Fig. 2

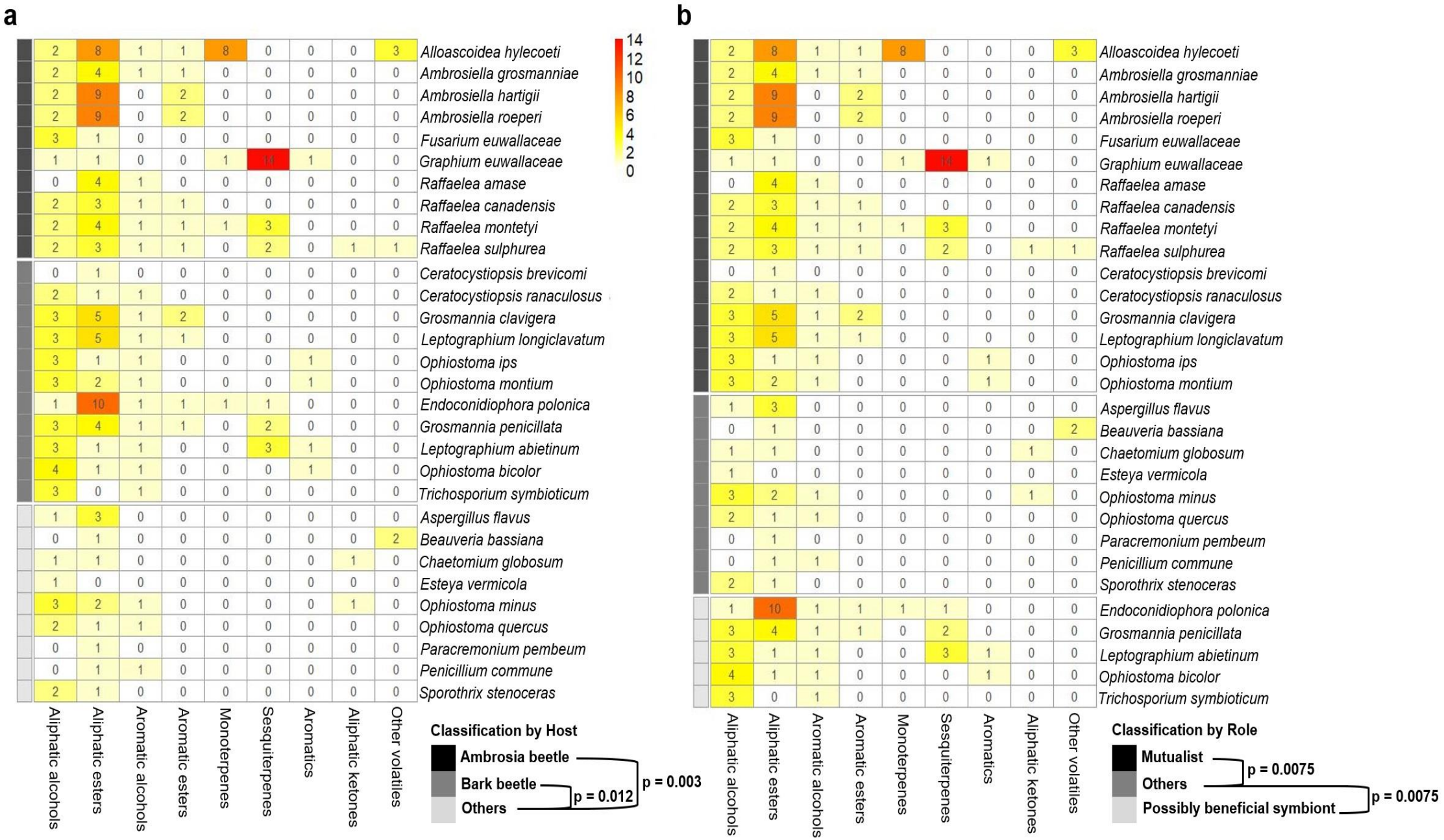
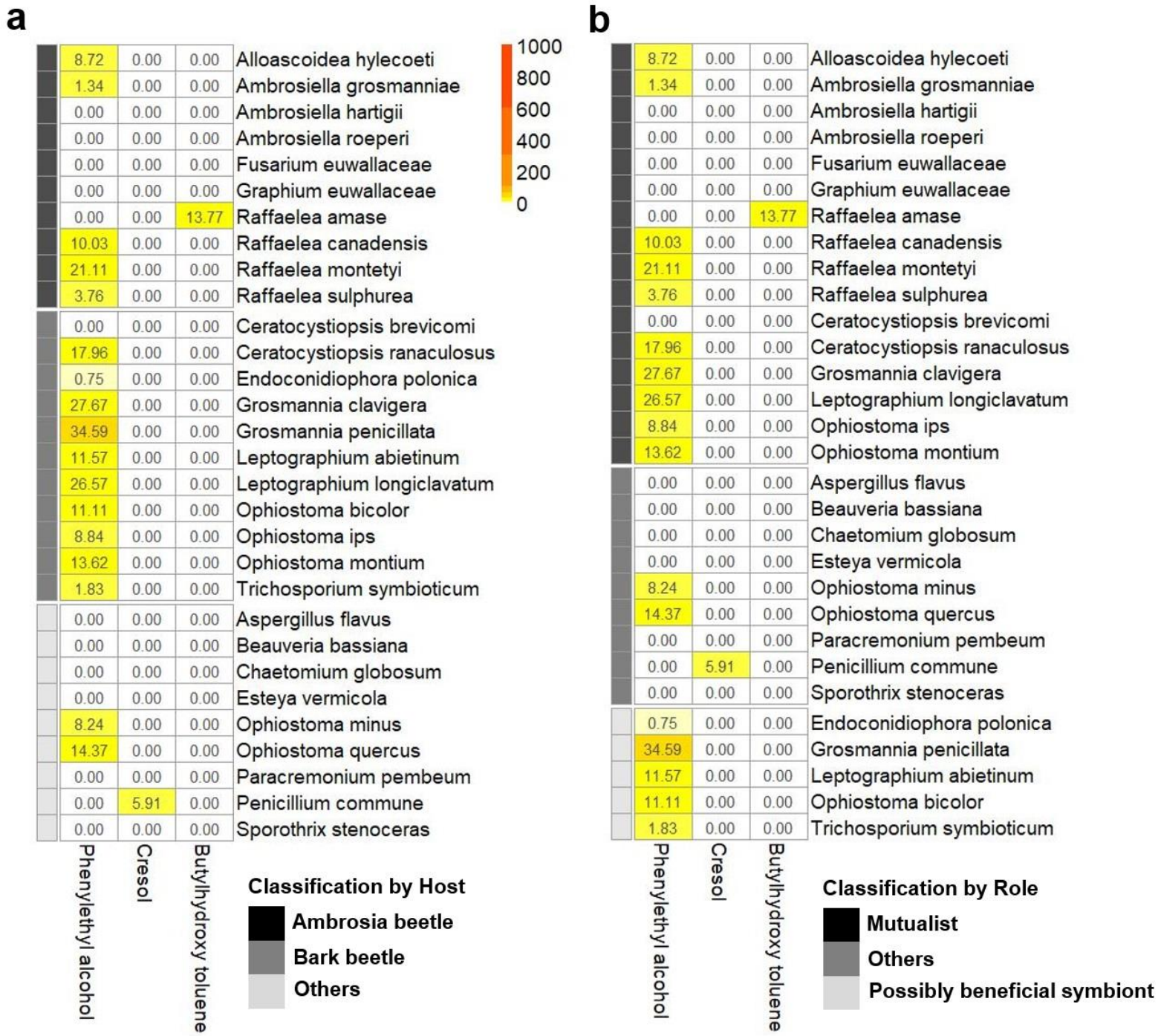

a
b
Alloascoidea hylecoeti
Ambrosiella grosmanniae
Ambrosiella hartigii
Ambrosiella roeperi
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Grosmannia clavigera
Leptographium longiclavatum
Ophiostoma ips
Ophiostoma montium
Endoconidiophora polonica
Grosmannia penicillata
Leptographium abietinum
Ophiostoma bicolor
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Aliphatic alcohols
Aliphatic esters
Aromatic alcohols
Aromatic esters
Monoterpenes
Sesquiterpenes
Aromatics
Aliphatic ketones
Other volatiles
14
12
10
8
6
4
2
0
Classification by Host
Ambrosia beetle
Bark beetle
Others
p = 0.012
p = 0.003
Classification by Role
Mutualist
Others
Possibly beneficial symbiont
p = 0.0075
p = 0.0075

Fig. 3


a
b
1000
800
600
400
200
0
Alloascoidea hylecoeti
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Ambrosiella hartigii
Ambrosiella roeperi
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Endoconidiophora polonica
Grosmannia clavigera
Grosmannia penicillata
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Leptographium longiclavatum
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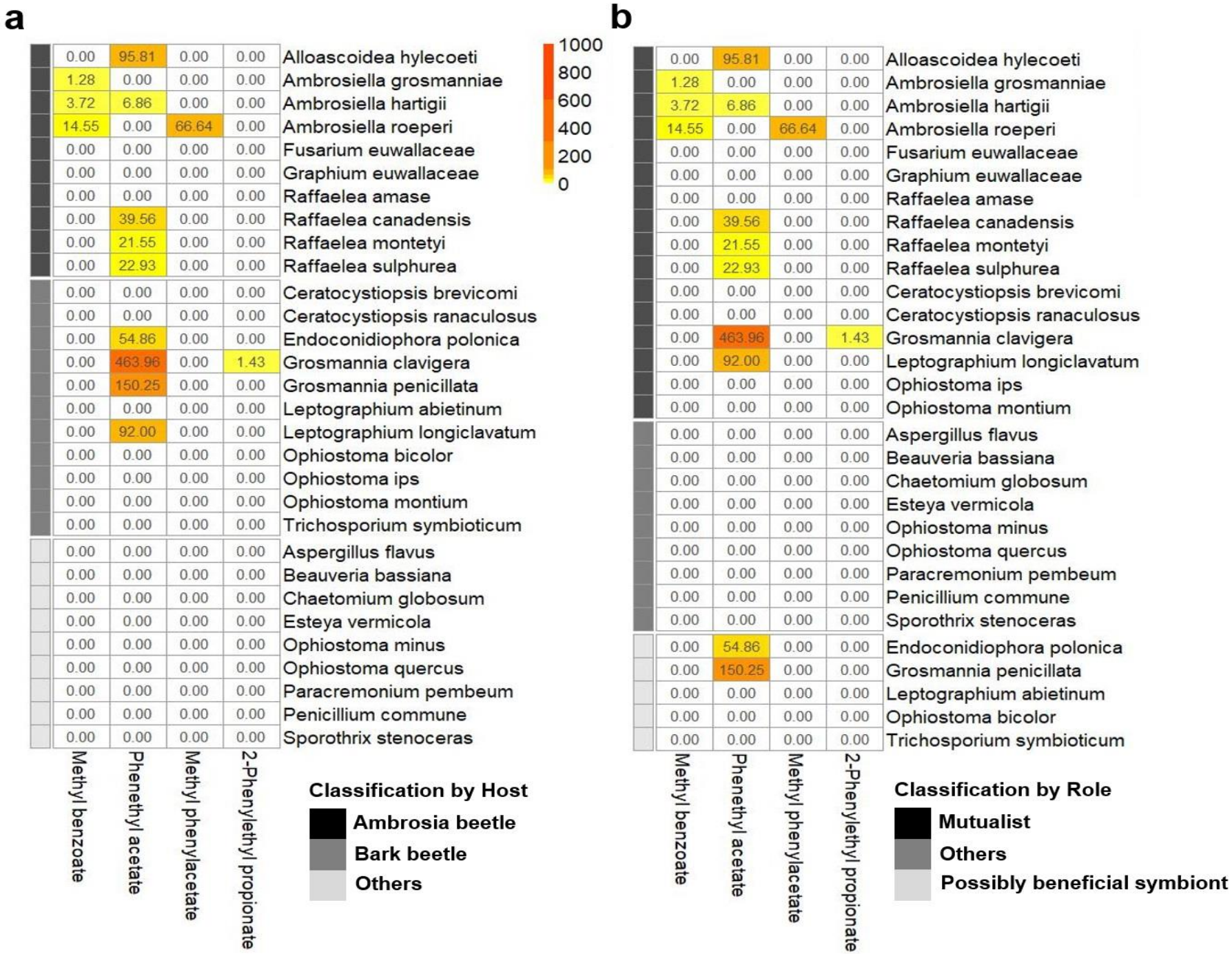
Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Phenylethyl alcohol
Cresol
Butylhydroxy toluene
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Fig. 4


a
b
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Alloascoidea hylecoeti
Ambrosiella grosmanniae
Ambrosiella hartigii
Ambrosiella roeperi
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Endoconidiophora polonica
Grosmannia clavigera
Grosmannia penicillata
Leptographium abietinum
Leptographium longiclavatum
Ophiostoma bicolor
Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Methyl benzoate
Phenethyl acetate
Methyl phenylacetate
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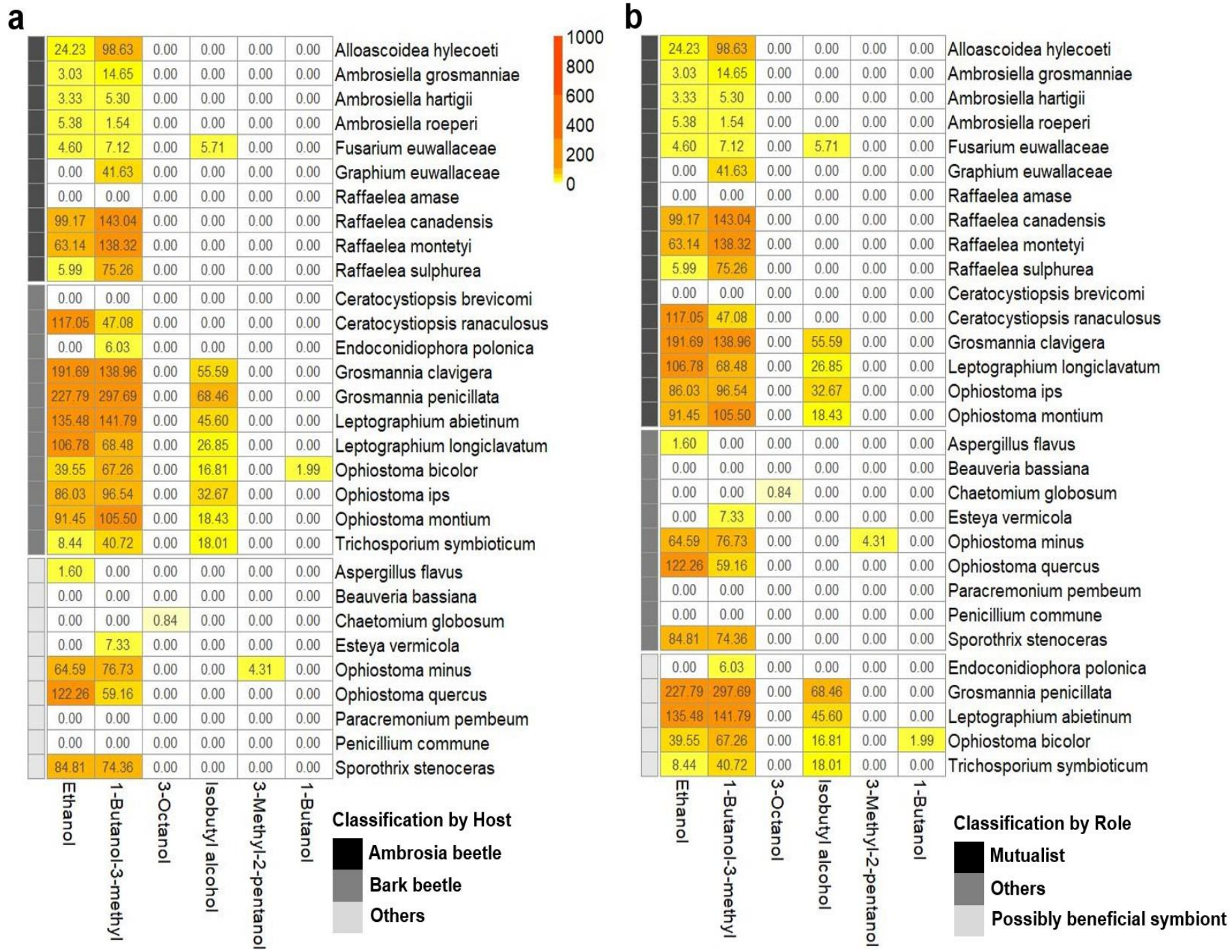
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Fig. 5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1000
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Alloascoidea hylecoeti
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Ambrosiella hartigii
Ambrosiella roeperi
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
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Raffaelea sulphurea
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Ceratocystiopsis ranaculosus
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Grosmannia penicillata
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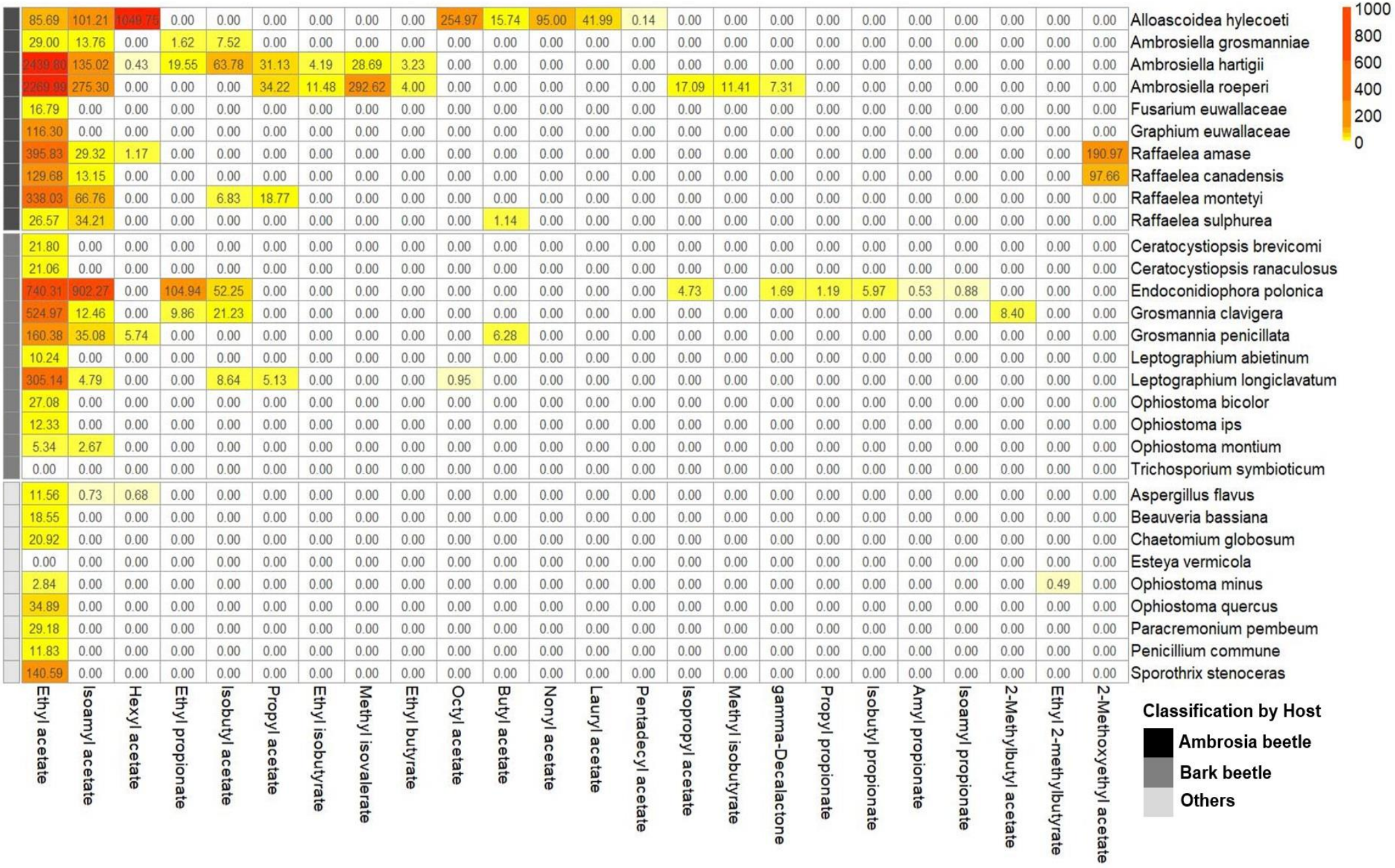
Leptographium longiclavatum
Ophiostoma bicolor
Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Ethanol
1-Butanol-3-methyl
3-Octanol
Isobutyl alcohol
3-Methyl-2-pentanol
1-Butanol
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Fig. 6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Ambrosiella hartigii
Ambrosiella roeperi
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Endoconidiophora polonica
Grosmannia clavigera
Grosmannia penicillata
Leptographium abietinum
Leptographium longiclavatum
Ophiostoma bicolor
Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Ethyl acetate
Isoamyl acetate
Hexyl acetate
Ethyl propionate
Isobutyl acetate
Propyl acetate
Ethyl isobutyrate
Methyl isovalerate
Ethyl butyrate
Octyl acetate
Butyl acetate
Nonyl acetate
Lauryl acetate
Pentadecyl acetate
Isopropyl acetate
Methyl isobutyrate
gamma-Decalactone
Propyl propionate
Isobutyl propionate
Amyl propionate
Isoamyl propionate
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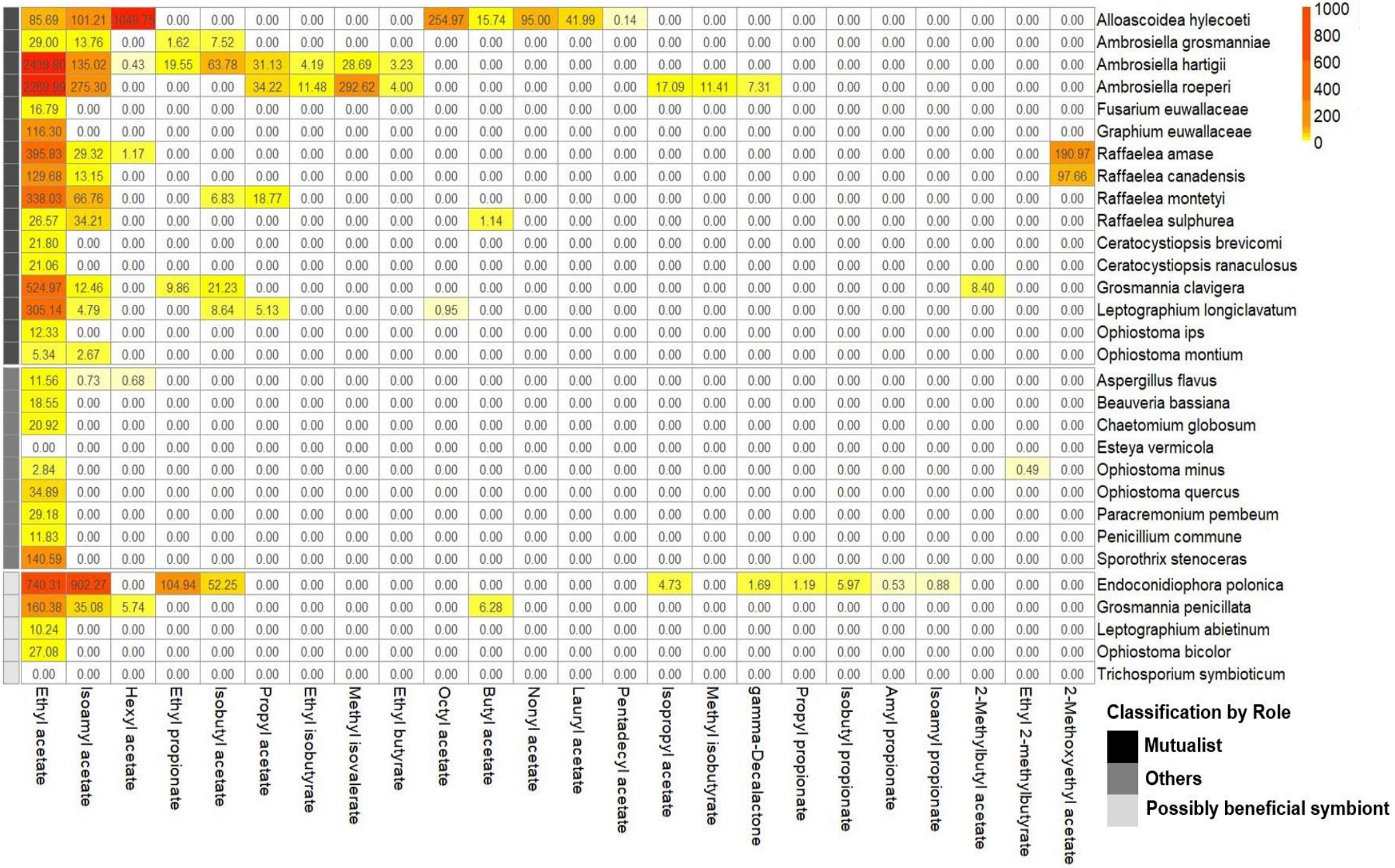
2-Methoxyethyl acetate
Classification by Host
Ambrosia beetle
Bark beetle
Others

Fig. 7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800
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200
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Alloascoidea hylecoeti
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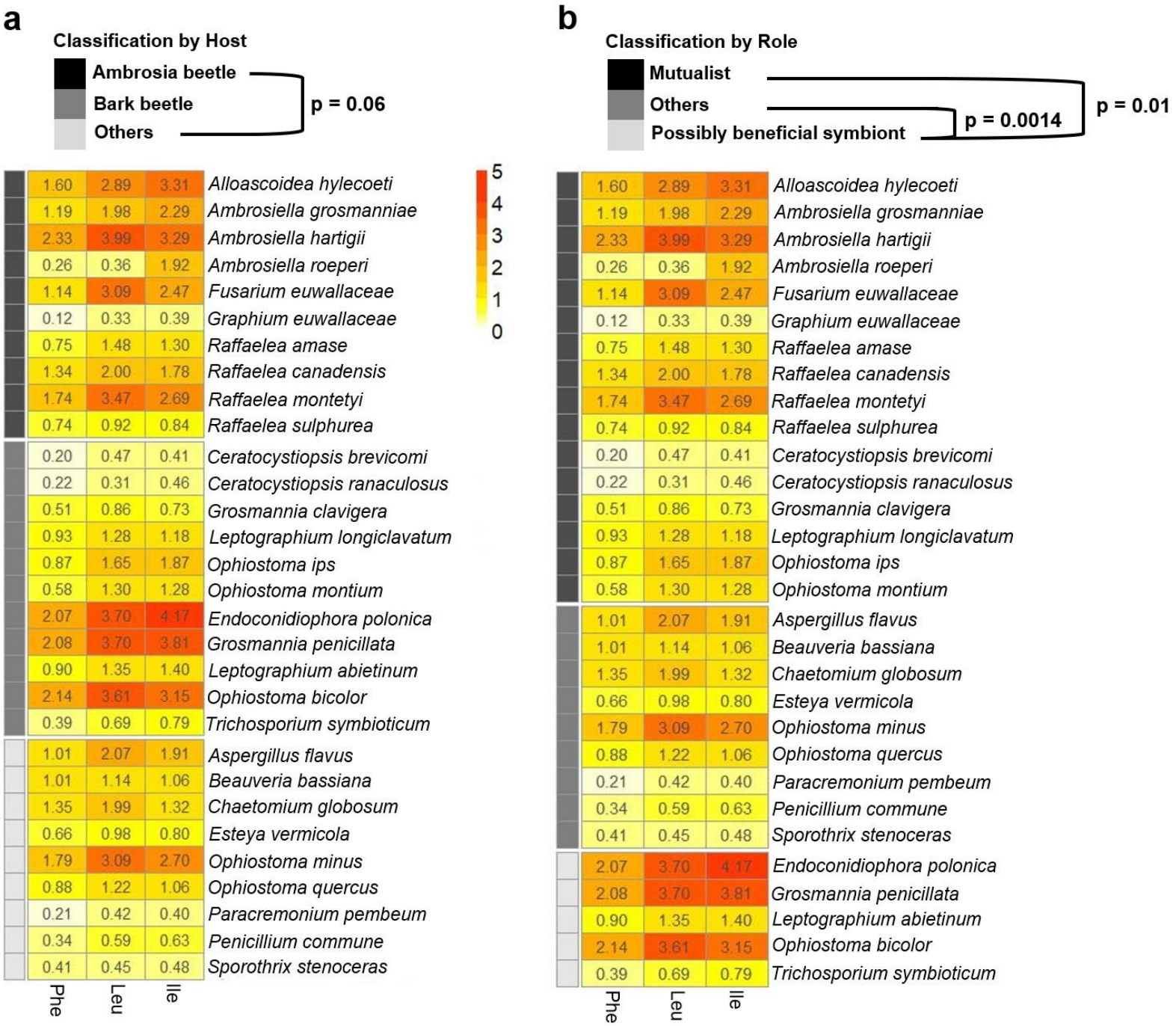
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Grosmannia clavigera
Leptographium longiclavatum
Ophiostoma ips
Ophiostoma montium
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Endoconidiophora polonica
Grosmannia penicillata
Leptographium abietinum
Ophiostoma bicolor
Trichosporium symbioticum
Ethyl acetate
Isoamyl acetate
Hexyl acetate
Ethyl propionate
Isobutyl acetate
Propyl acetate
Ethyl isobutyrate
Methyl isovalerate
Ethyl butyrate
Octyl acetate
Butyl acetate
Nonyl acetate
Lauryl acetate
Pentadecyl acetate
Isopropyl acetate
Methyl isobutyrate
gamma-Decalactone
Propyl propionate
Isobutyl propionate
Amyl propionate
Isoamyl propionate
2-Methylbutyl acetate
Ethyl 2-methylbutyrate
2-Methoxyethyl acetate
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Fig. 8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Classification by Host
Ambrosia beetle
Bark beetle
Others
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b
Classification by Role
Mutualist
Others
Possibly beneficial symbiont
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p = 0.01
5
4
3
2
1
0
1.60 2.89 3.31 Alloascoidea hylecoeti
1.19 1.98 2.29 Ambrosiella grosmanniae
2.33 3.99 3.29 Ambrosiella hartigii
0.26 0.36 1.92 Ambrosiella roeperi
1.14 3.09 2.47 Fusarium euwallaceae
0.12 0.33 0.39 Graphium euwallaceae
0.75 1.48 1.30 Raffaelea amase
1.34 2.00 1.78 Raffaelea canadensis
1.74 3.47 2.69 Raffaelea montetyi
0.74 0.92 0.84 Raffaelea sulphurea
0.20 0.47 0.41 Ceratocystiopsis brevicomi
0.22 0.31 0.46 Ceratocystiopsis ranaculosus
0.51 0.86 0.73 Grosmannia clavigera
0.93 1.28 1.18 Leptographium longiclavatum
0.87 1.65 1.87 Ophiostoma ips
0.58 1.30 1.28 Ophiostoma montium
2.07 3.70 4.17 Endoconidiophora polonica
2.08 3.70 3.81 Grosmannia penicillata
0.90 1.35 1.40 Leptographium abietinum
2.14 3.61 3.15 Ophiostoma bicolor
0.39 0.69 0.79 Trichosporium symbioticum
1.01 2.07 1.91 Aspergillus flavus
1.01 1.14 1.06 Beauveria bassiana
1.35 1.99 1.32 Chaetomium globosum
0.66 0.98 0.80 Esteya vermicola
1.79 3.09 2.70 Ophiostoma minus
0.88 1.22 1.06 Ophiostoma quercus
0.21 0.42 0.40 Paracremonium pembeum
0.34 0.59 0.63 Penicillium commune
0.41 0.45 0.48 Sporothrix stenoceras
Phe Leu Ile
1.60 2.89 3.31 Alloascoidea hylecoeti
1.19 1.98 2.29 Ambrosiella grosmanniae
2.33 3.99 3.29 Ambrosiella hartigii
0.26 0.36 1.92 Ambrosiella roeperi
1.14 3.09 2.47 Fusarium euwallaceae
0.12 0.33 0.39 Graphium euwallaceae
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1.34 2.00 1.78 Raffaelea canadensis
1.74 3.47 2.69 Raffaelea montetyi
0.74 0.92 0.84 Raffaelea sulphurea
0.20 0.47 0.41 Ceratocystiopsis brevicomi
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0.93 1.28 1.18 Leptographium longiclavatum
0.87 1.65 1.87 Ophiostoma ips
0.58 1.30 1.28 Ophiostoma montium
1.01 2.07 1.91 Aspergillus flavus
1.01 1.14 1.06 Beauveria bassiana
1.35 1.99 1.32 Chaetomium globosum
0.66 0.98 0.80 Esteya vermicola
1.79 3.09 2.70 Ophiostoma minus
0.88 1.22 1.06 Ophiostoma quercus
0.21 0.42 0.40 Paracremonium pembeum
0.34 0.59 0.63 Penicillium commune
0.41 0.45 0.48 Sporothrix stenoceras
2.07 3.70 4.17 Endoconidiophora polonica
2.08 3.70 3.81 Grosmannia penicillata
0.90 1.35 1.40 Leptographium abietinum
2.14 3.61 3.15 Ophiostoma bicolor
0.39 0.69 0.79 Trichosporium symbioticum
Phe Leu Ile

Table 1: Overview of the 30 fungal isolates used in this study and additional information on the phylogenetic order, classification (by host and by role), source of isolation, and literature.

Fungal Species	**Phylogenetic order**	**Classification by host**	**Classification by role**	**Source of isolation**	**References**
Raffaelea sulphurea	Ophiostomatales (Clade 1)	Ambrosia Beetle	Mutualist	*Xyleborinus saxesenii*	[1]
R. montetyi	Ophiostomatales (Clade 1)	Ambrosia Beetle	Mutualist	*Platypus cylindrus*	[2]
R. amase	Ophiostomatales (Clade 1)	Ambrosia Beetle	Mutualist	*Amasa concitatus*	[3]
Esteya vermicola	Ophiostomatales (Clade 1)	Free-living	None	None	[4]
R. canadensis	Ophiostomatales (Clade 2)	Ambrosia Beetle	Mutualist	*X. saxesenii*	[5]
Grosmannia penicillata	Ophiostomatales (Clade 3)	Bark Beetle	Possibly beneficial symbiont	*Ips* spp.	[6]
Leptographium abietinum	Ophiostomatales (Clade 3)	Bark Beetle	Possibly beneficial symbiont	*Dendroctonus rufipennis*	[7]
G. clavigera	Ophiostomatales (Clade 3)	Bark Beetle	Mutualist	*D. ponderosae*	[8]
L. longiclavatum	Ophiostomatales (Clade 3)	Bark Beetle	Mutualist	*D. ponderosae*	[9]
Ceratocystiopsis brevicomis	Ophiostomatales (Clade 4)	Bark Beetle	Mutualist	*D. brevicomis*	[10]
Sporothrix stenoceras	Ophiostomatales (Clade 4)	Various insects	Role unknown	None	[11]
C. ranaculosus	Ophiostomatales (Clade 4)	Bark Beetle	Mutualist	*D. frontalis*	[12]
Ophiostoma bicolor	Ophiostomatales (Clade 5)	Bark Beetle	Possibly beneficial symbiont	*Ips* spp.	[13]
O. quercus	Ophiostomatales (Clade 5)	Various insects	Role unknown	*Platypus* spp.	[14]
Trichosporium symbioticum	Ophiostomatales (Clade 5)	Bark Beetle	Possibly beneficial symbiont	*Scolytus ventralis*	[15]
O. ips	Ophiostomatales (Clade 5)	Bark Beetle	Mutualist	*I. pini*	[16]
O. minus	Ophiostomatales (Clade 5)	Various bark	Antagonist to *D. frontalis*	*D. frontalis*	[17]

		beetles	and *D. brevicomis*		
O. montium	Ophiostomatales (Clade 5)	Bark Beetle	Mutualist	*D. ponderosae*	[18]
Endoconidiophora polonica	Microascales (Clade 1)	Bark Beetle	Possibly beneficial symbiont	*Ips typographus*	[19]
Ambrosiella grosmanniae	Microascales (Clade 1)	Ambrosia Beetle	Mutualist	*Xylosandrus germanus*	[20]
A. roeperi	Microascales (Clade 1)	Ambrosia Beetle	Mutualist	*X. crassisculus*	[21]
A. hartigii	Microascales (Clade 1)	Ambrosia Beetle	Mutualist	*Anisandrus dispar*	[5]
Graphium euwallaceae	Microascales (Clade 2)	Ambrosia Beetle	Mutualist	*Euwallacea fornicatus*	[22]
Paracremonium pembeum	Hypocreales	Ambrosia Beetle	Role unknown	*E. fornicatus*	[22]
Beauveria bassiana	Hypocreales	Various insects	Antagonist	None	[23 - 24]
Fusarium euwallaceae	Hypocreales	Ambrosia Beetle	Mutualist	*E. fornicatus*	[25]
Alloascoidea hylecoeti	Saccaromycetales	Ambrosia Beetle	Mutualist	*Hylecoetus dermestoides*	[5]
Penicillium commune	Eurotiales	Various insects	Role unknown	*X. saxesenii*	[26 – 28]
Aspergillus flavus	Eurotiales	Various insects	Antagonist	*X. saxesenii*	[27 - 29]
Chaetomium globosum	Sordariales	Various insects	Antagonist	*X. saxesenii*	[27, 28, 30]

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Annex – Supplementary Figures

Figure Legends

Fig. S1: Heatmap visualizing the absolute number of identified volatiles (N = 76) per examined fungus (N = 30) after a cultivation time of 12 days, which were classified into nine different chemical groups (including the group “other volatiles”). Each number represents the absolute number of volatiles produced by one fungus within each chemical group. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]). We indicated significant differences within each classification system by adding p-values to the legend of a and b, which we obtained from a NMDS analysis.

Fig. S2: Heatmap visualizing the concentration of each volatile (N = 3) classified into the group of aromatic alcohols per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S3: Heatmap visualizing the concentration of each volatile (N = 3) classified into the group of aromatic esters per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two

classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S4: Heatmap visualizing the concentration of each volatile (N = 7) classified into the group of aliphatic alcohols per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S5: Heatmap visualizing the concentration of each volatile (N = 18) classified into the group of aliphatic esters per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S6: Heatmap visualizing the concentration of each volatile (N = 3) classified into the group of aliphatic ketones per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are

indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S7: Heatmap visualizing the concentration of each volatile (N = 3) classified into the group of aliphatic ketones per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S8: Heatmap visualizing the concentration of each volatile (N = 2) classified into the group of aromatics per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S9: Heatmap visualizing the concentration of each volatile (N = 2) classified into the group of aromatics per examined fungus (N = 30) after a cultivation time of 12

days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S10: Heatmap visualizing the concentration of each volatile (N = 10) classified into the group of monoterpenes per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S11: Heatmap visualizing the concentration of each volatile (N = 10) classified into the group of monoterpenes per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S12: Heatmap visualizing the concentration of each volatile (N = 23) classified into the group of sesquiterpenes per examined fungus (N = 30) after a cultivation time of 6 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S13: Heatmap visualizing the concentration of each volatile (N = 23) classified into the group of sesquiterpenes per examined fungus (N = 30) after a cultivation time of 12 days in counts/mm2 (semi-quantitative pattern). Values within the heatmap are indicating the individual mean volatile concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]).

Fig. S14: Heatmap visualizing the absolute concentration of the three amino acids phenylalanine (Phe), leucine (Leu), and isoleucine (Ile) within each examined fungus (N = 30) after a cultivation time of 12 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Two classification systems were applied to all examined fungi based on (a) the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) as well as on (b) the role of each fungus (mutualist, possibly beneficial symbiont, and

others [the latest including the classifications “role unknown” and “antagonists”]). No statistical differences within each classification could be detected.

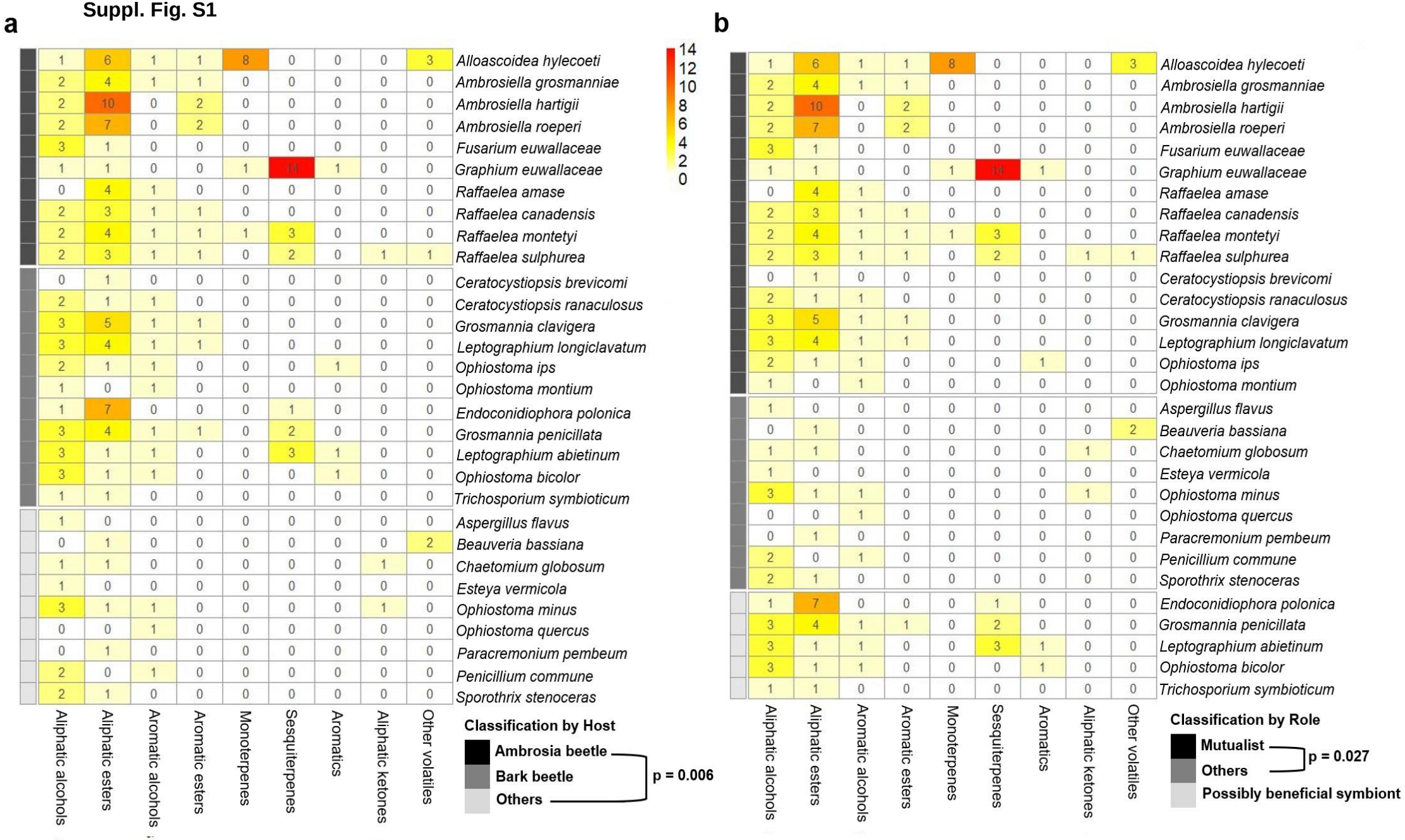
Fig. S15: Heatmap visualizing the absolute concentration of 18 free amino acids within each examined fungus (N = 30) after a cultivation time of 6 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Here, the classification system based on the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) is shown. We indicated significant differences by adding p-values to the legend, which we obtained from a NMDS analysis.

Fig. S16: Heatmap visualizing the absolute concentration of 18 free amino acids within each examined fungus (N = 30) after a cultivation time of 6 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Here, the classification system based on the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]) is shown. We indicated significant differences by adding p-values to the legend, which we obtained from a NMDS analysis.

Fig. S17: Heatmap visualizing the absolute concentration of 18 free amino acids within each examined fungus (N = 30) after a cultivation time of 12 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Here, the classification system based on the associated host (ambrosia beetle, bark beetle, and others [the latest including the classifications “various insects” and “free-living”]) is shown. We indicated significant differences by adding p-values to the legend, which we obtained from a NMDS analysis.

Fig. S18: Heatmap visualizing the absolute concentration of 18 free amino acids within each examined fungus (N = 30) after a cultivation time of 12 days in nmol/mg = µmol/g (quantitative pattern). Values within the heatmap are indicating the individual mean amino acid concentration produced by each fungus. Here, the classification system based on the role of each fungus (mutualist, possibly beneficial symbiont, and others [the latest including the classifications “role unknown” and “antagonists”]) is shown. No statistical differences could be detected.

Suppl. Fig. S1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Graphium euwallaceae
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Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Grosmannia clavigera
Leptographium longiclavatum
Ophiostoma ips
Ophiostoma montium
Endoconidiophora polonica
Grosmannia penicillata
Leptographium abietinum
Ophiostoma bicolor
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Aliphatic alcohols
Aliphatic esters
Aromatic alcohols
Aromatic esters
Monoterpenes
Sesquiterpenes
Aromatics
Aliphatic ketones
Other volatiles
Classification by Host
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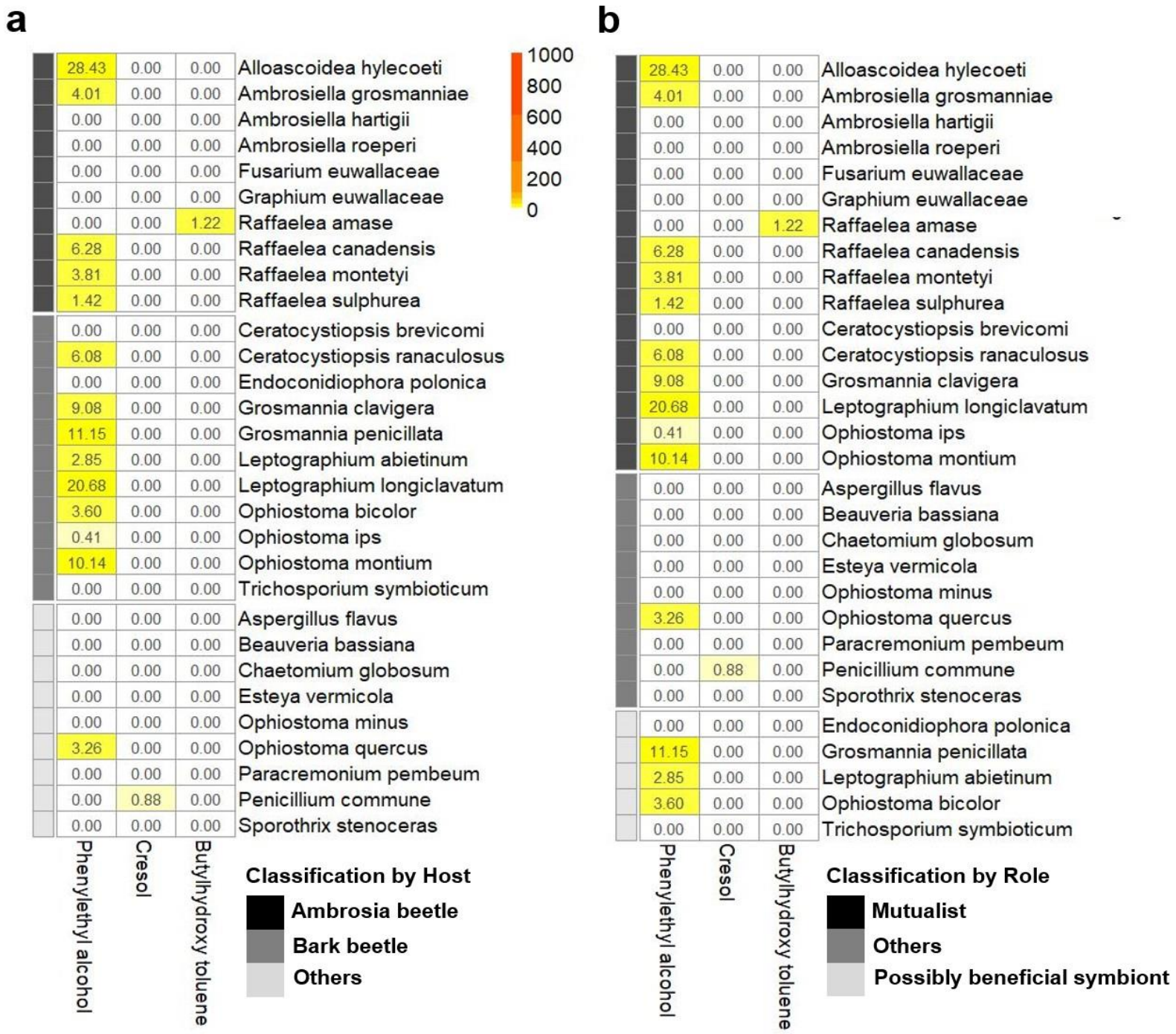
Bark beetle
Others
p = 0.006
Classification by Role
Mutualist
Others
Possibly beneficial symbiont
p = 0.027

Suppl. Fig. S2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Graphium euwallaceae
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Raffaelea montetyi
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Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Phenylethyl alcohol
Cresol
Butylhydroxy toluene
Classification by Host
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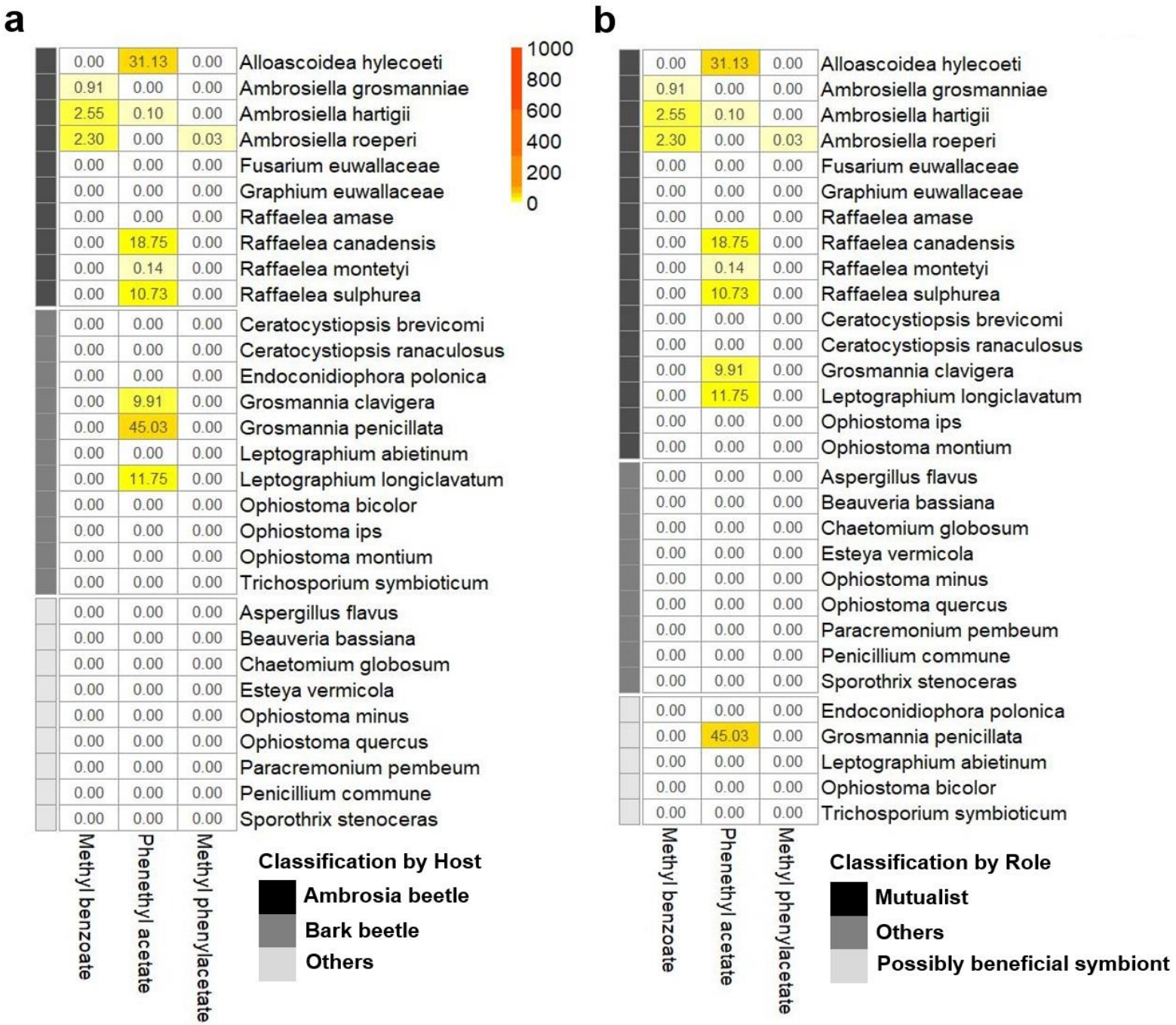
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S3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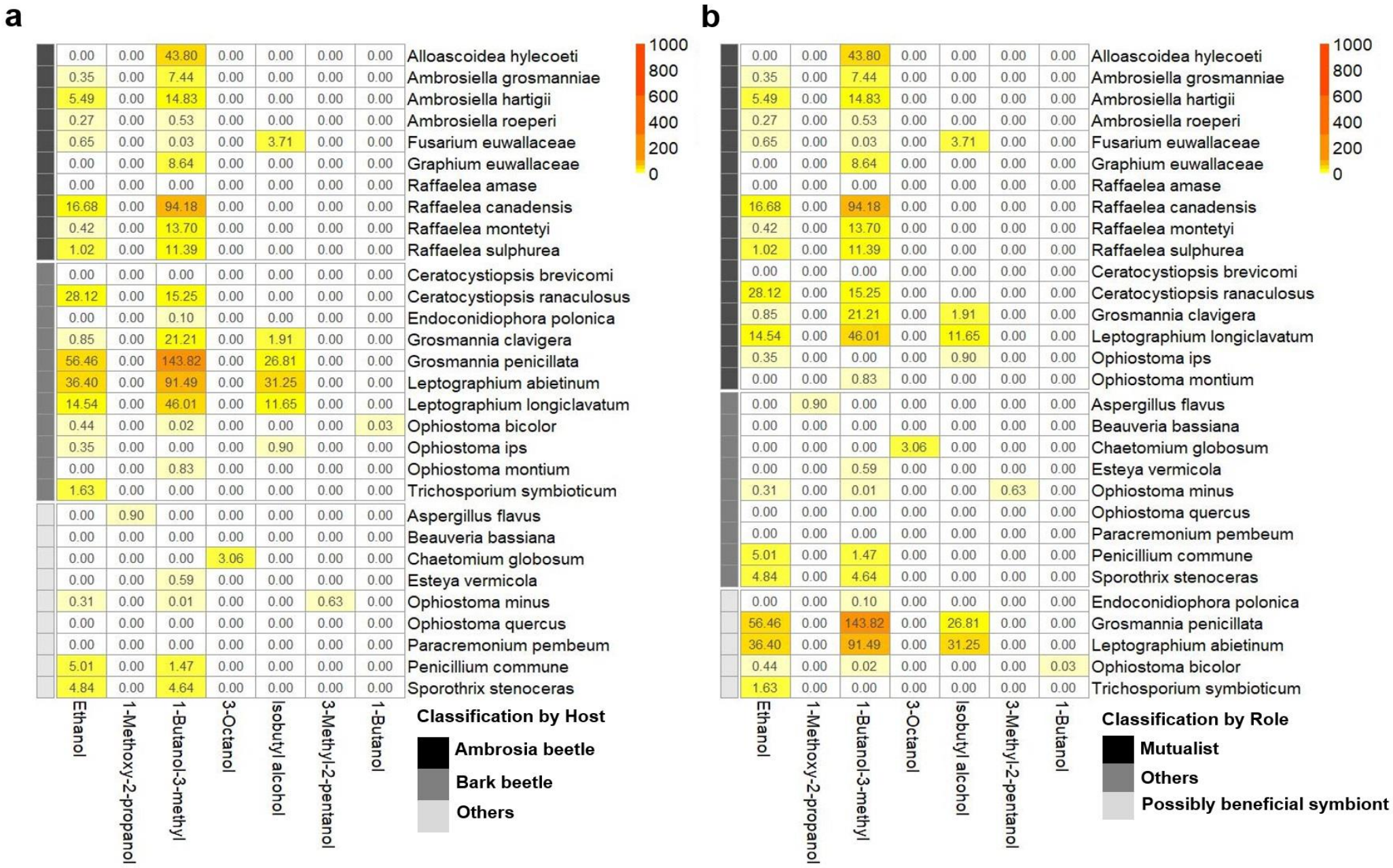
Fusarium euwallaceae
Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Endoconidiophora polonica
Grosmannia clavigera
Grosmannia penicillata
Leptographium abietinum
Leptographium longiclavatum
Ophiostoma bicolor
Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Methyl benzoate
Phenethyl acetate
Methyl phenylacetate
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S4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Ambrosiella roeperi
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Graphium euwallaceae
Raffaelea amase
Raffaelea canadensis
Raffaelea montetyi
Raffaelea sulphurea
Ceratocystiopsis brevicomi
Ceratocystiopsis ranaculosus
Endoconidiophora polonica
Grosmannia clavigera
Grosmannia penicillata
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Ophiostoma montium
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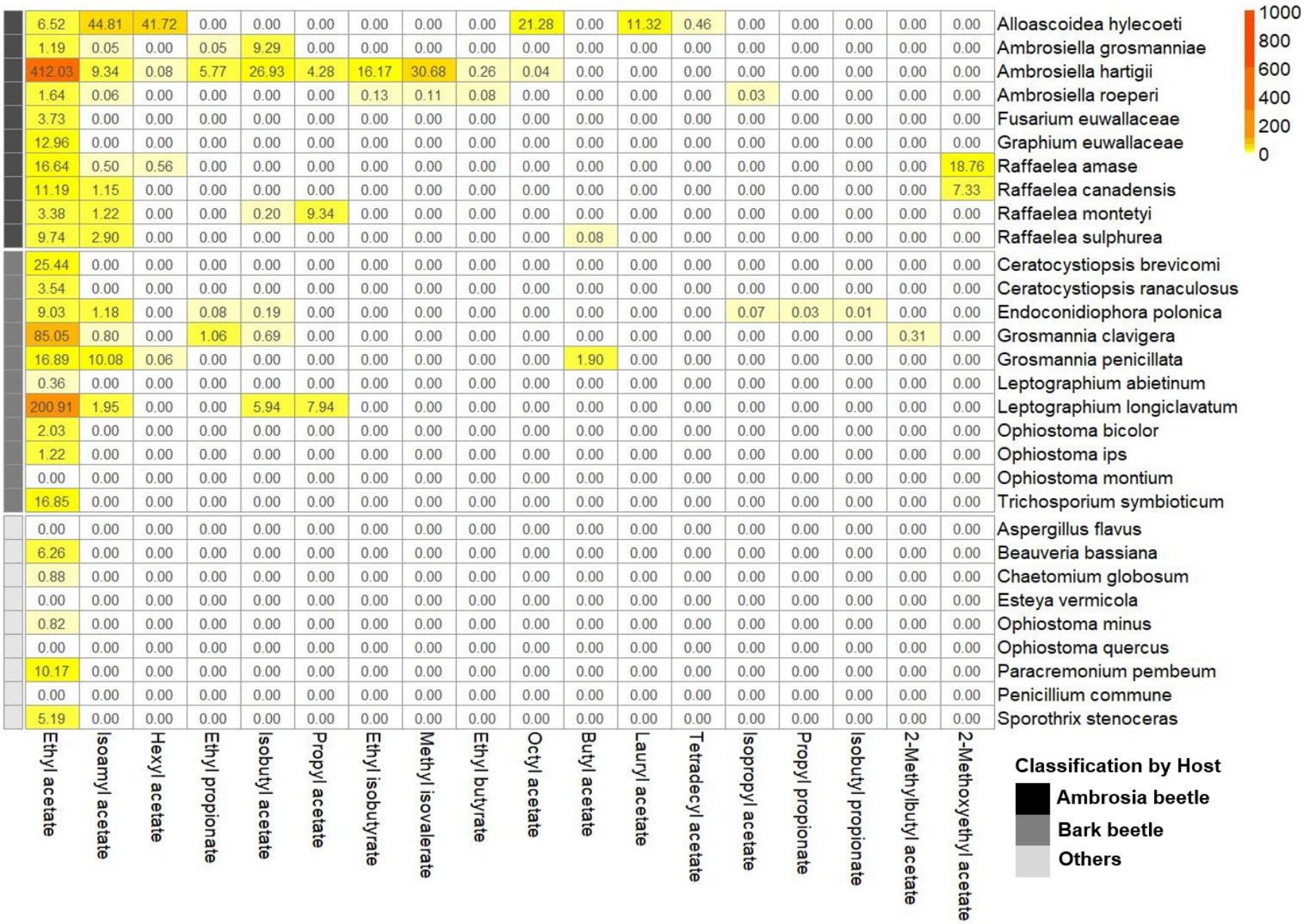
Beauveria bassiana
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Ethanol
1-Methoxy-2-propanol
1-Butanol-3-methyl
3-Octanol
Isobutyl alcohol
3-Methyl-2-pentanol
1-Butanol
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S5a



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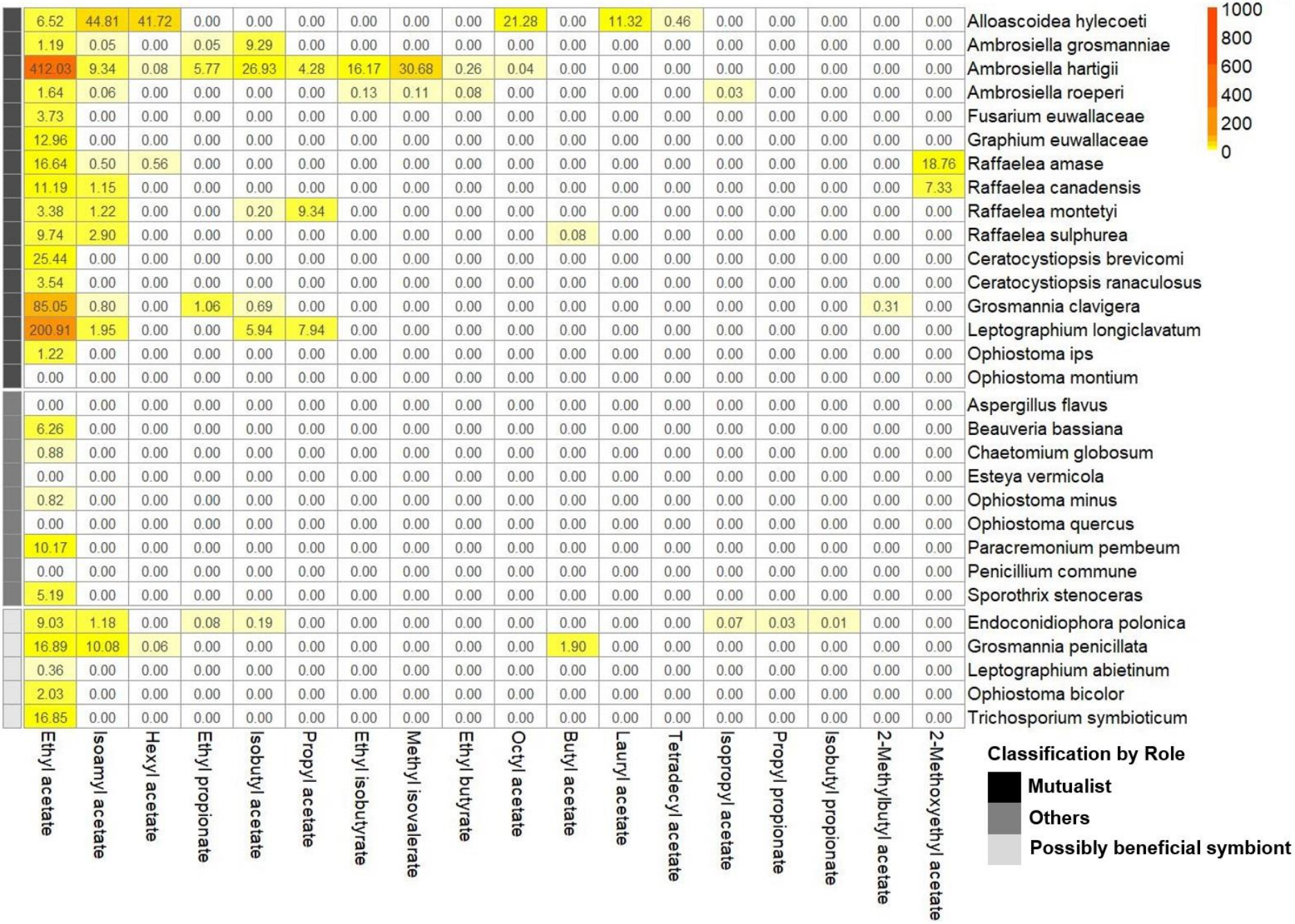

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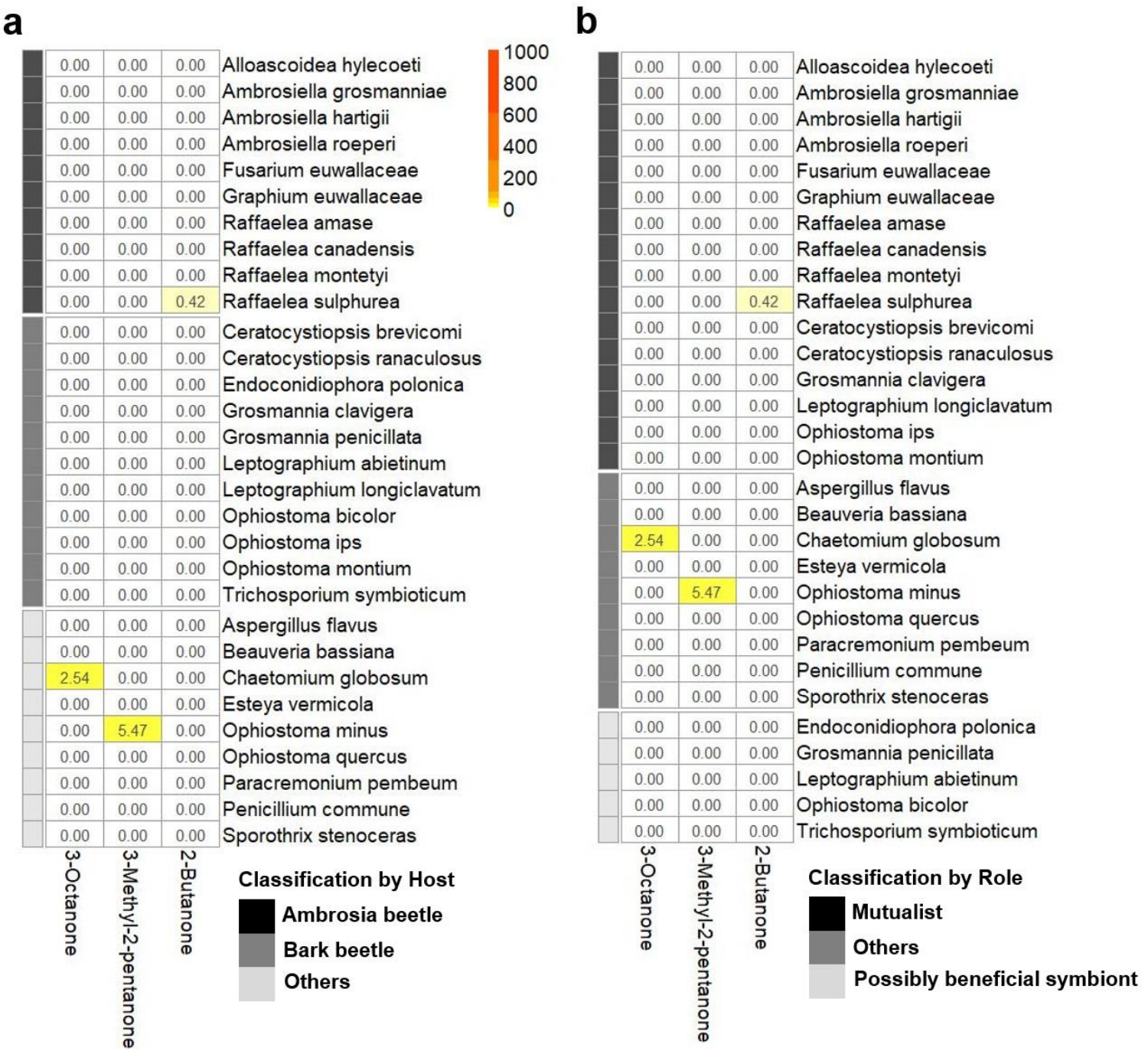
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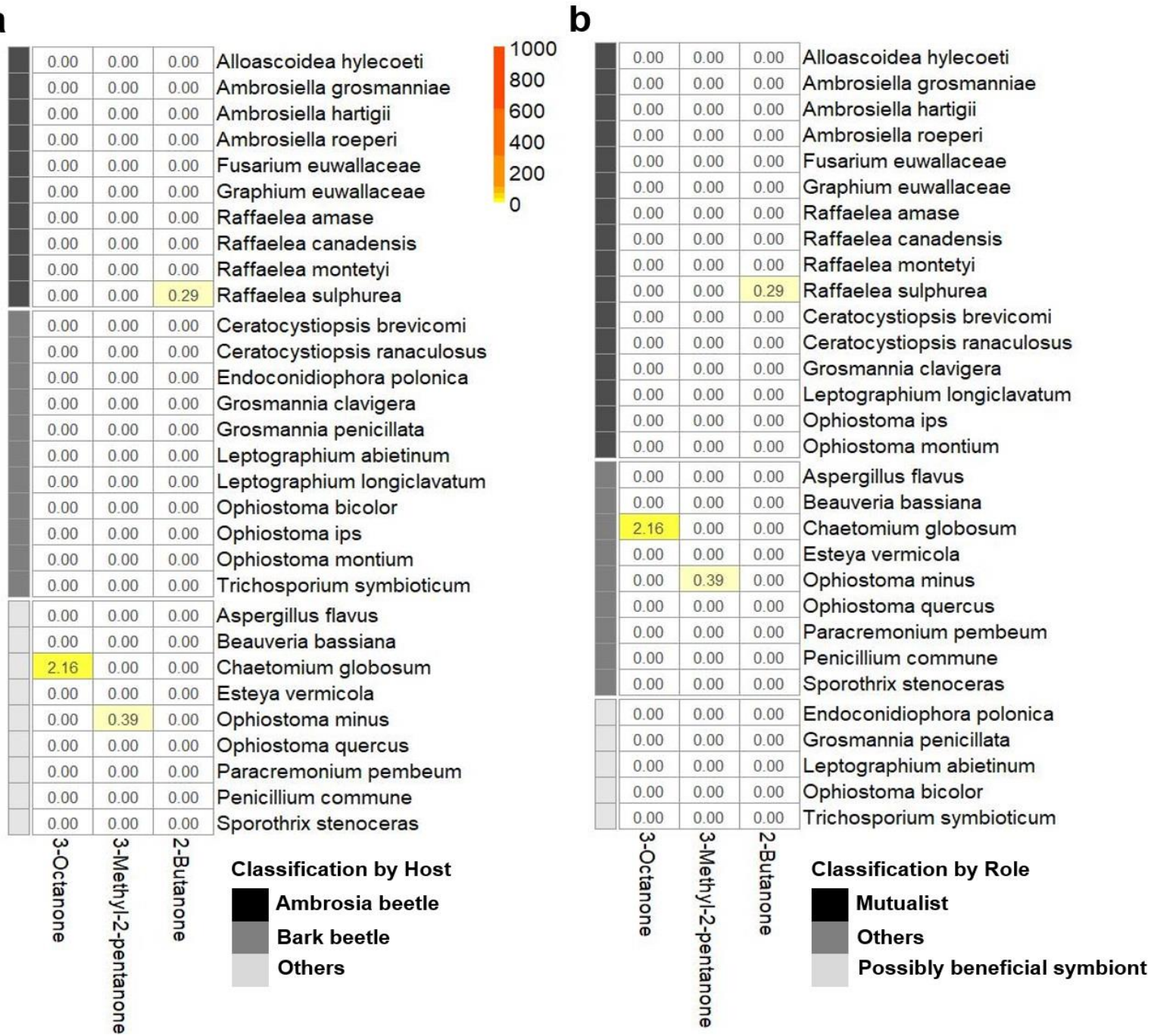
Chaetomium globosum
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
3-Octanone
3-Methyl-2-pentanone
2-Butanone
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S7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Fusarium euwallaceae
Graphium euwallaceae
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Ophiostoma minus
Ophiostoma quercus
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Penicillium commune
Sporothrix stenoceras
3-Octanone
3-Methyl-2-pentanone
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Classification by Host
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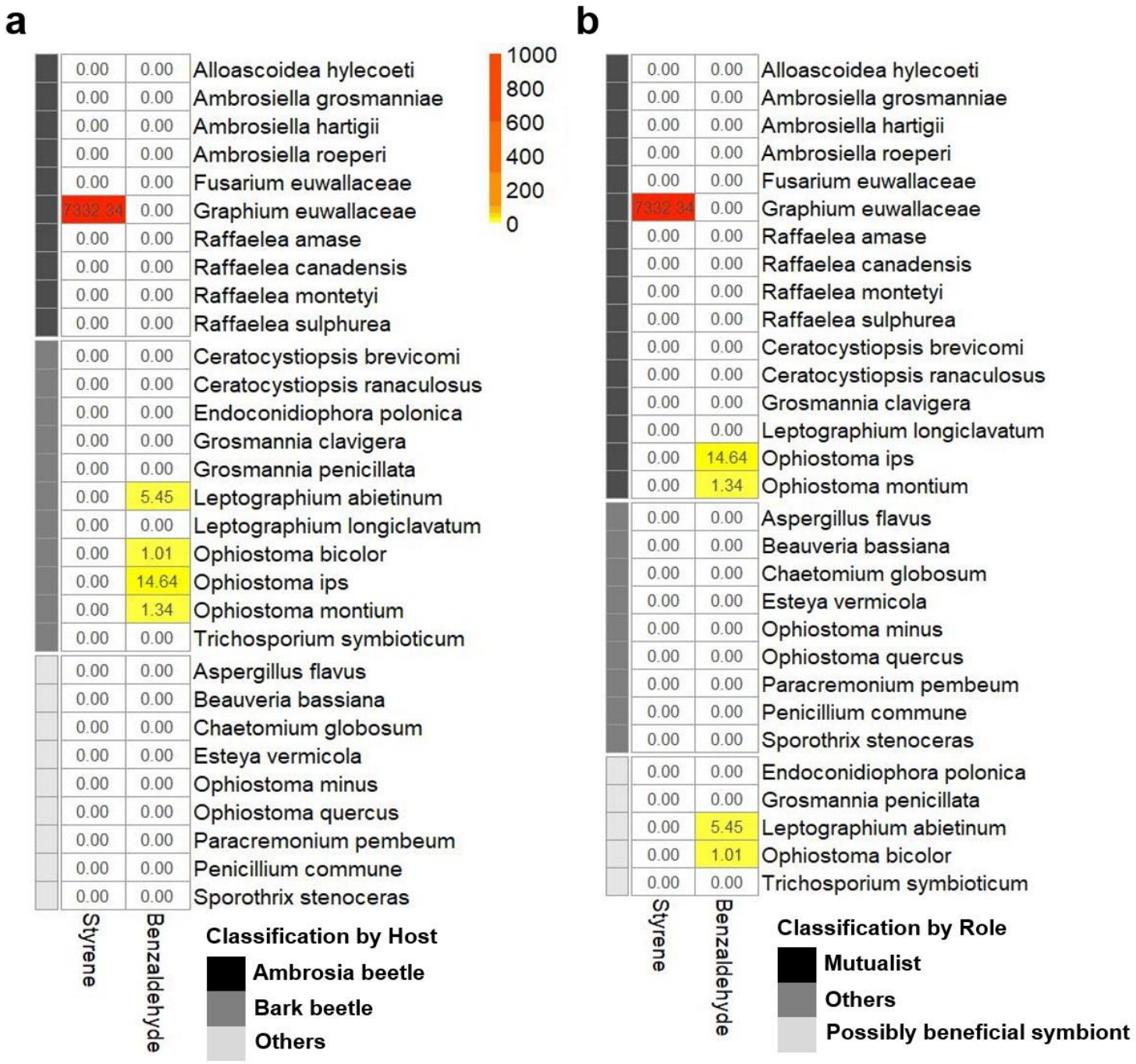
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S8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Raffaelea montetyi
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Ceratocystiopsis ranaculosus
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Aspergillus flavus
Beauveria bassiana
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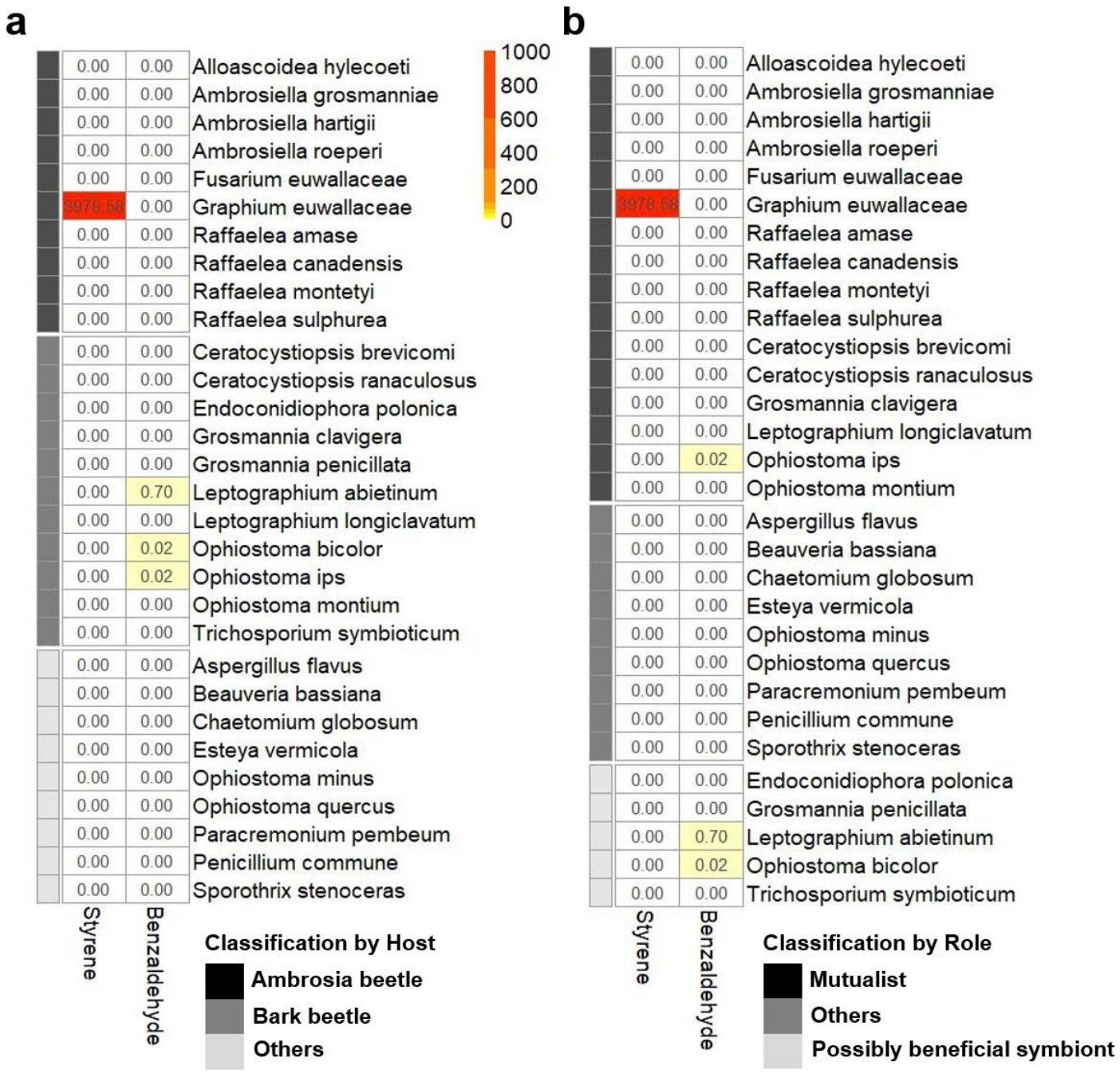
Esteya vermicola
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
Styrene
Benzaldehyde
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

Suppl. Fig. S9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Ophiostoma ips
Ophiostoma montium
Trichosporium symbioticum
Aspergillus flavus
Beauveria bassiana
Chaetomium globosum
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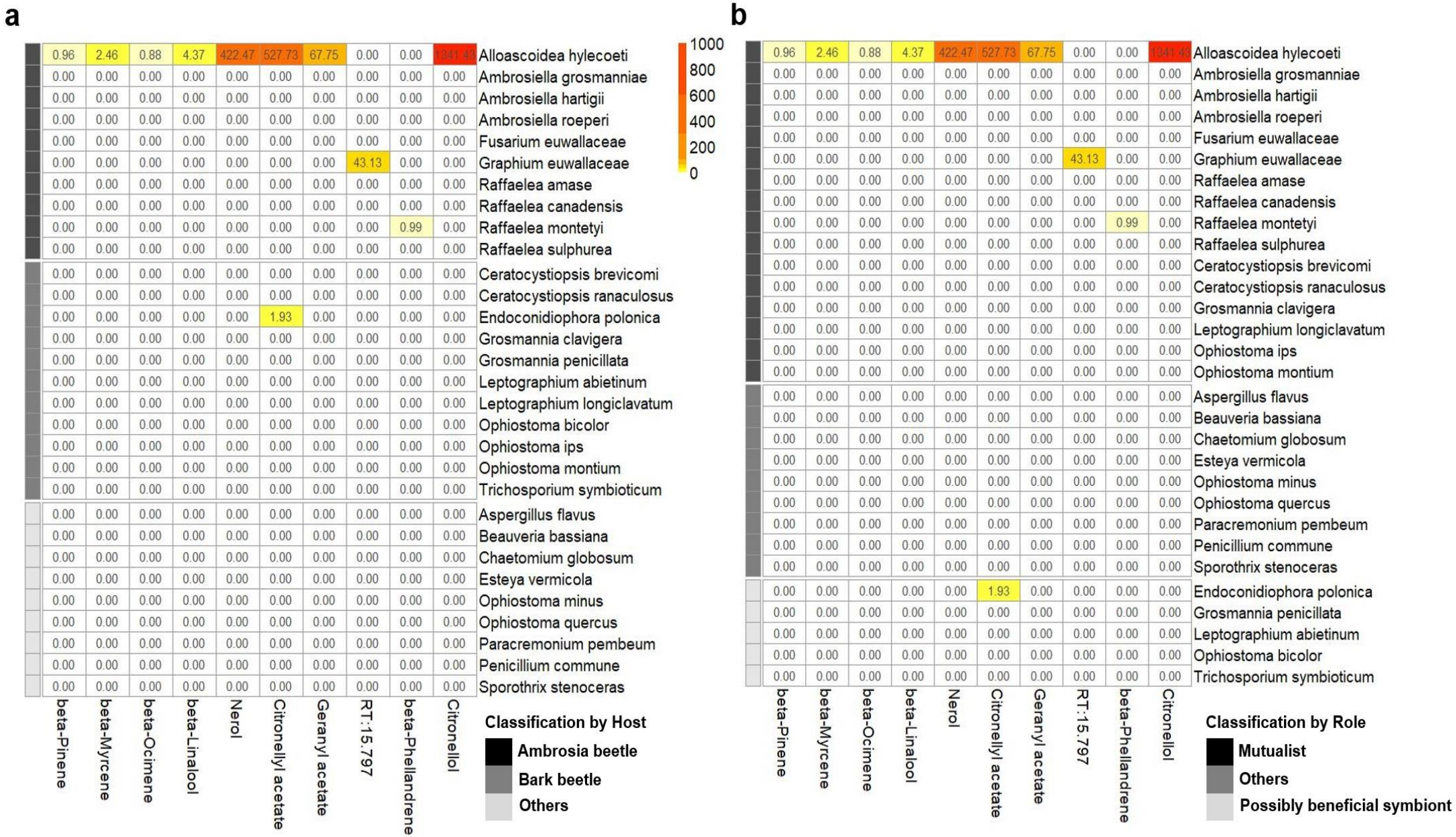
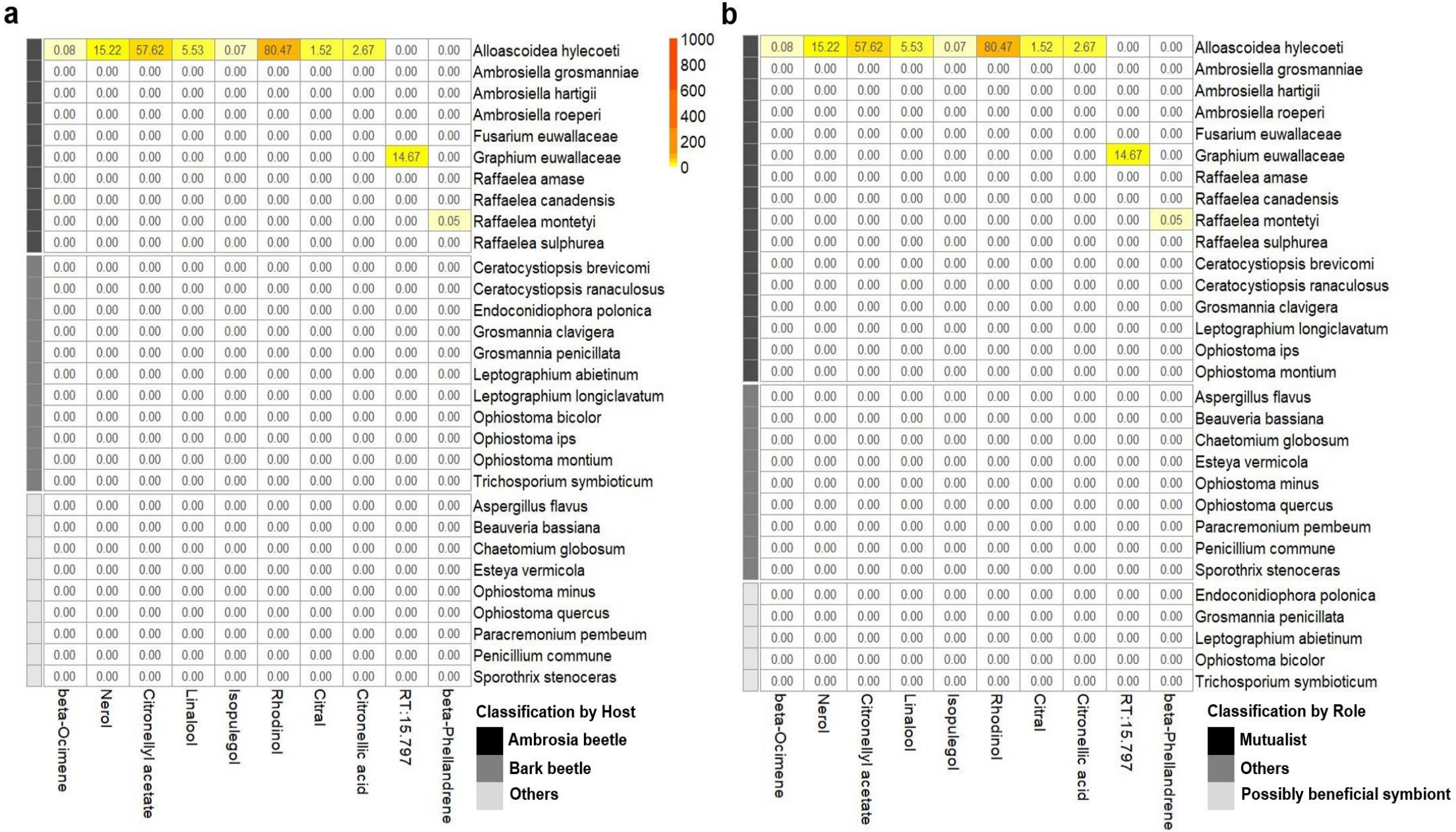
Ophiostoma minus
Ophiostoma quercus
Paracremonium pembeum
Penicillium commune
Sporothrix stenoceras
3978.58
Styrene
Benzaldehyde
Classification by Host
Ambrosia beetle
Bark beetle
Others
Classification by Role
Mutualist
Others
Possibly beneficial symbiont

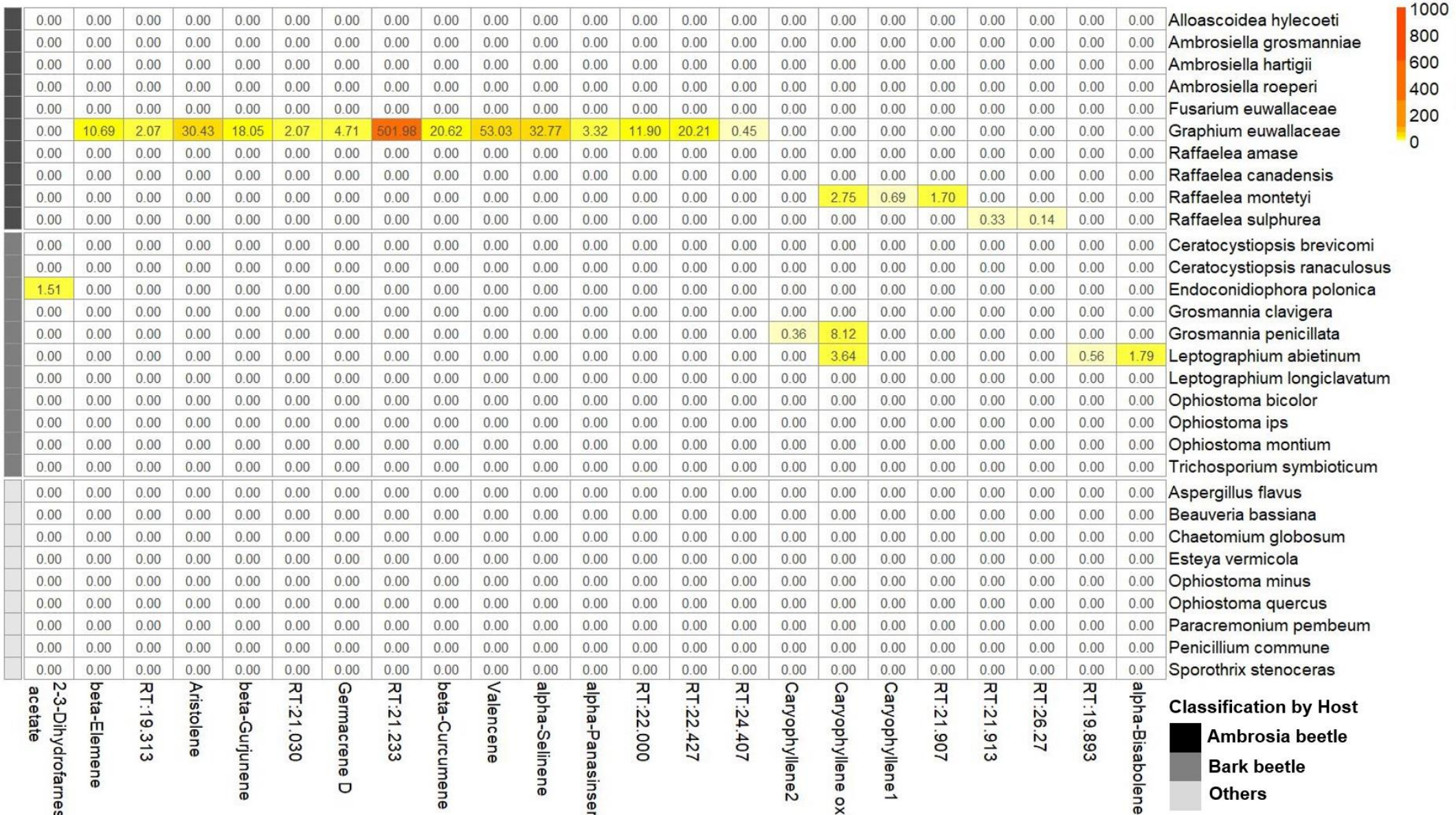
Suppl. Fig. S10



Suppl. Fig. S11



Suppl. Fig. S12a



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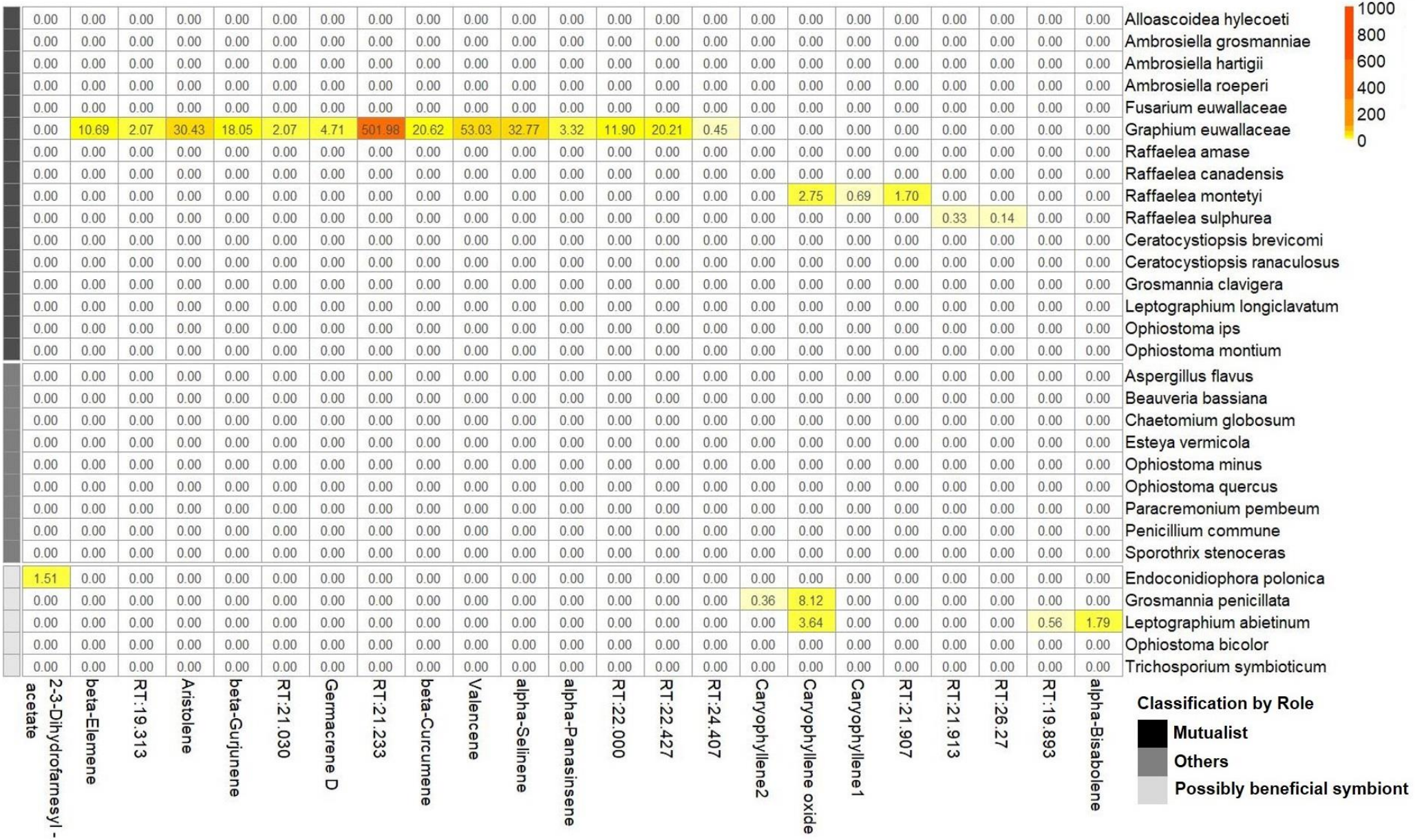

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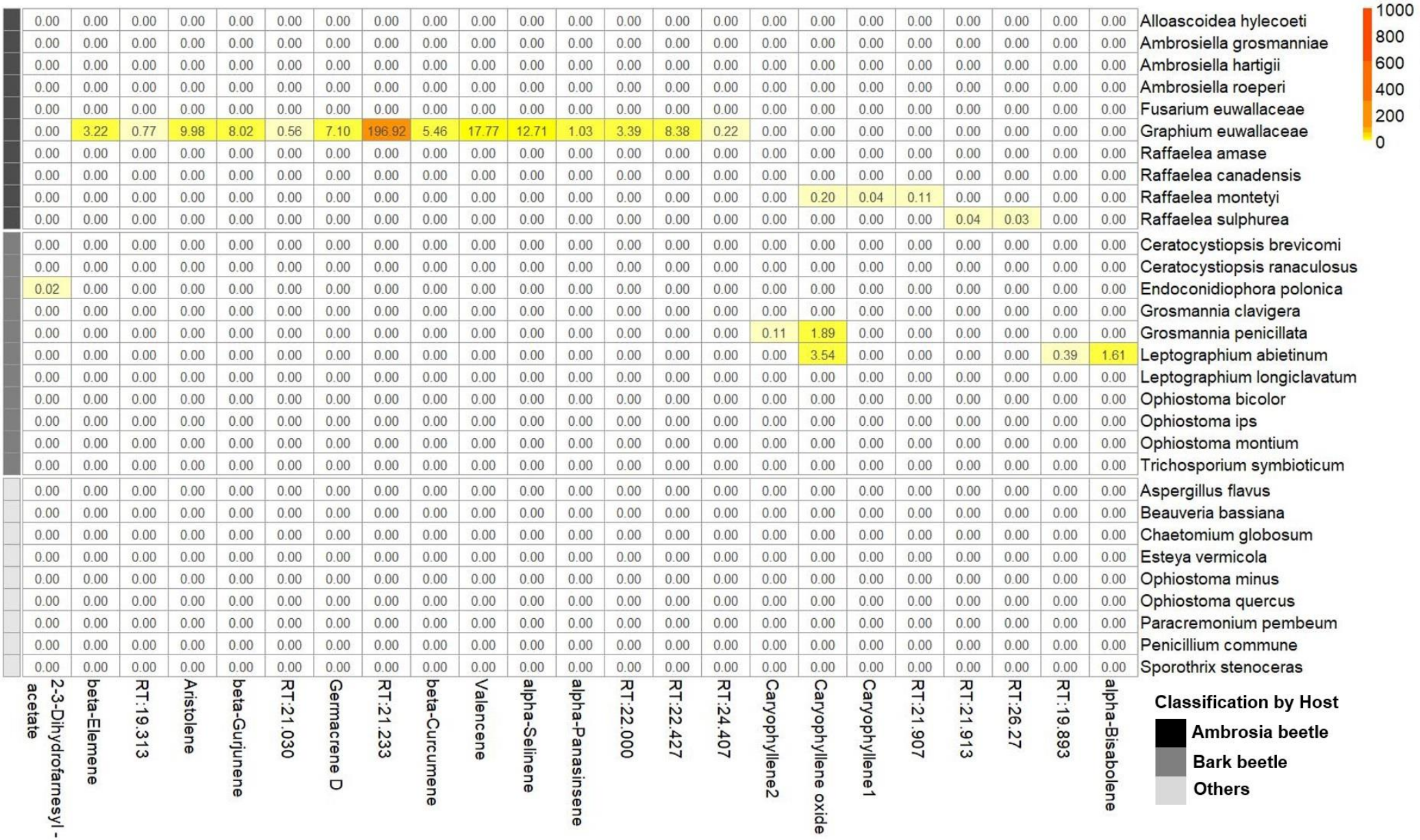

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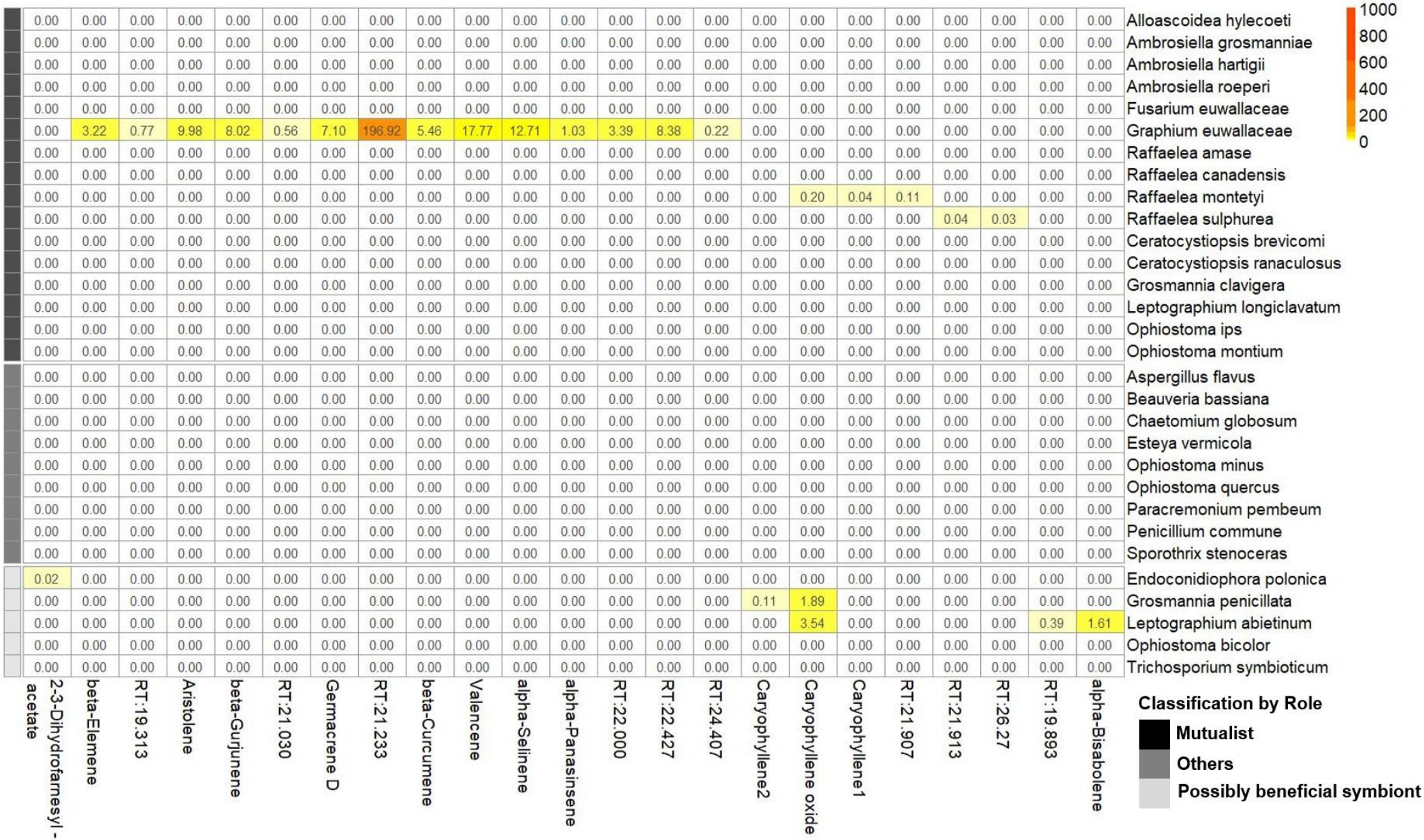

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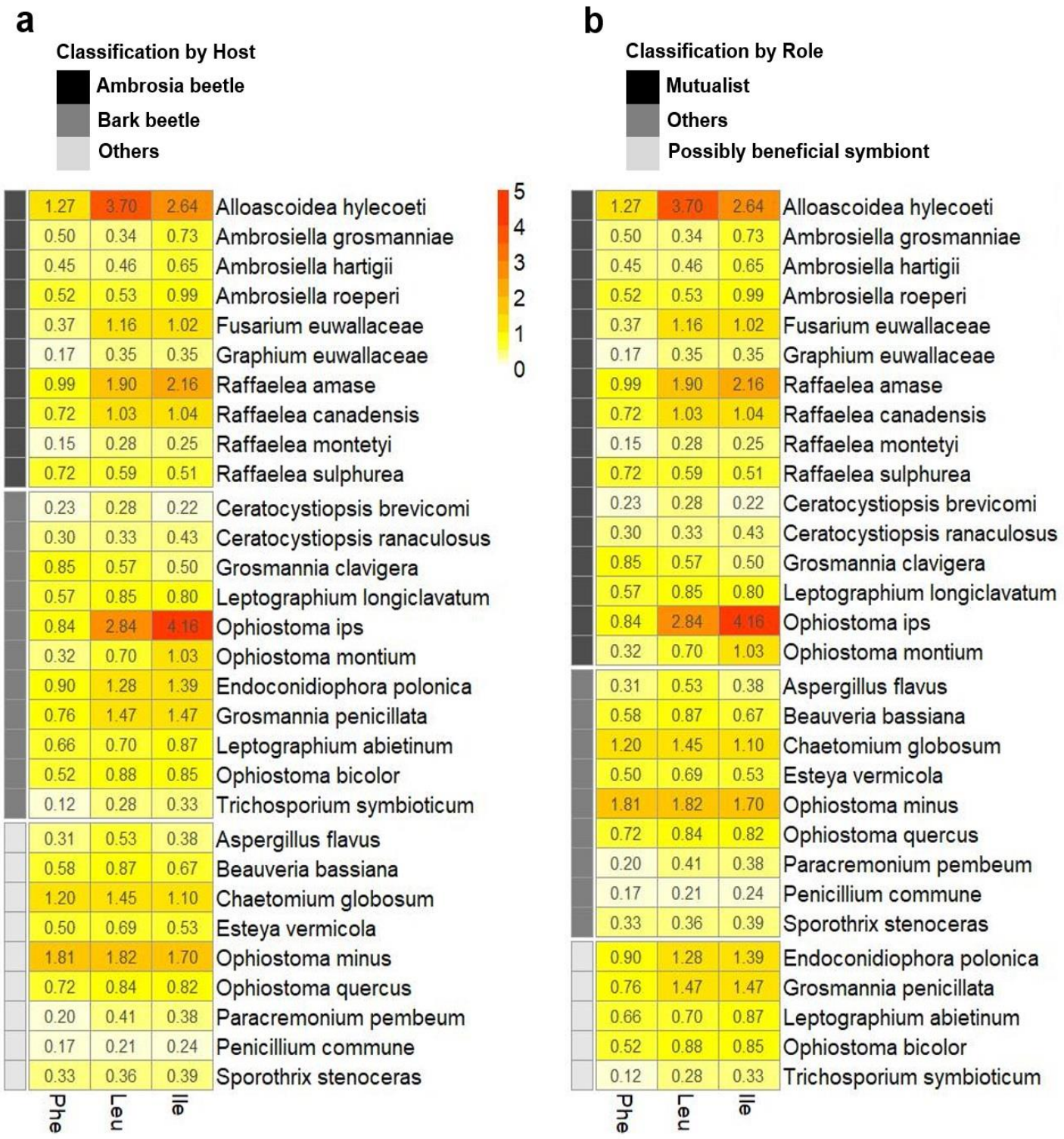

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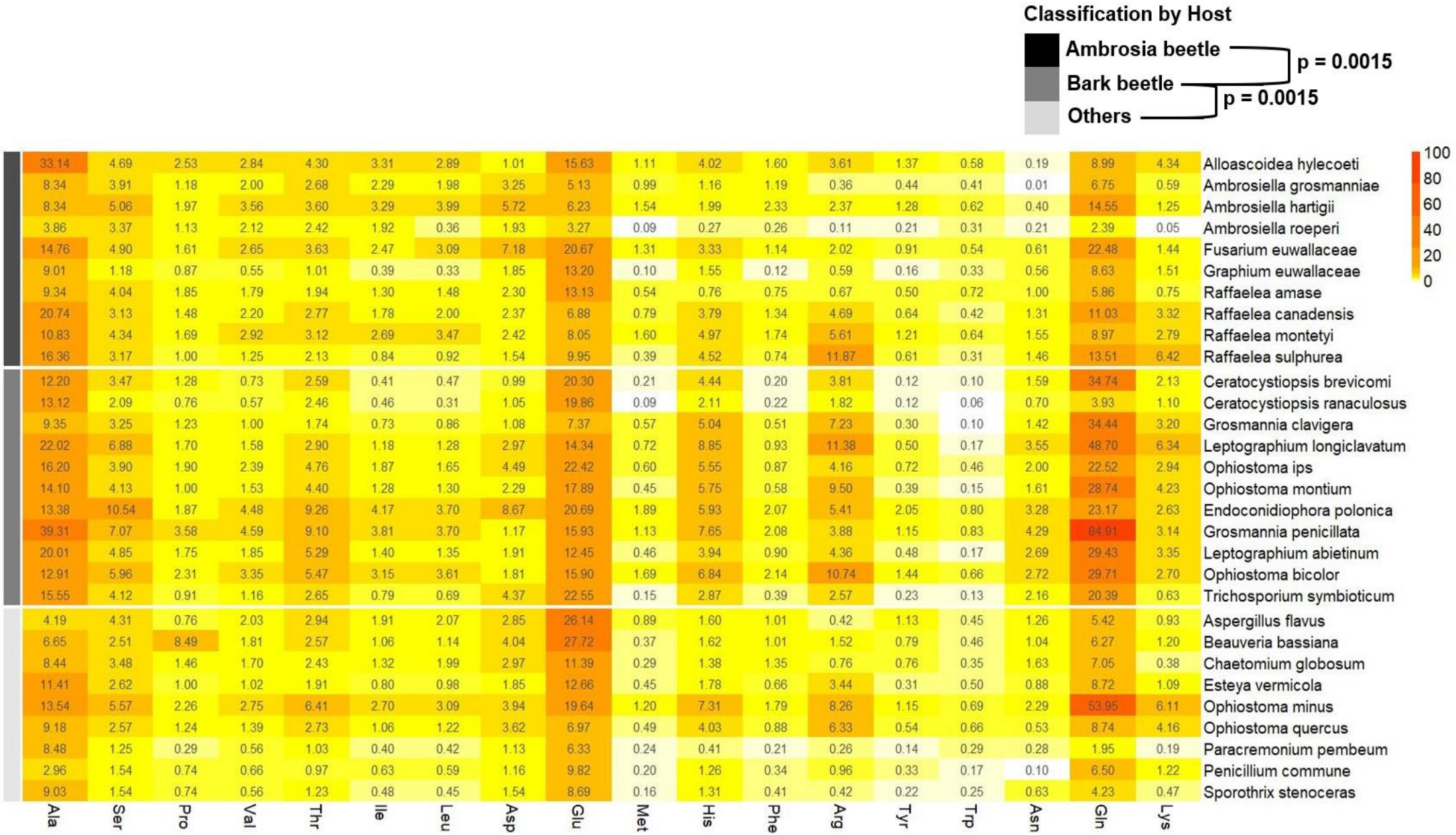

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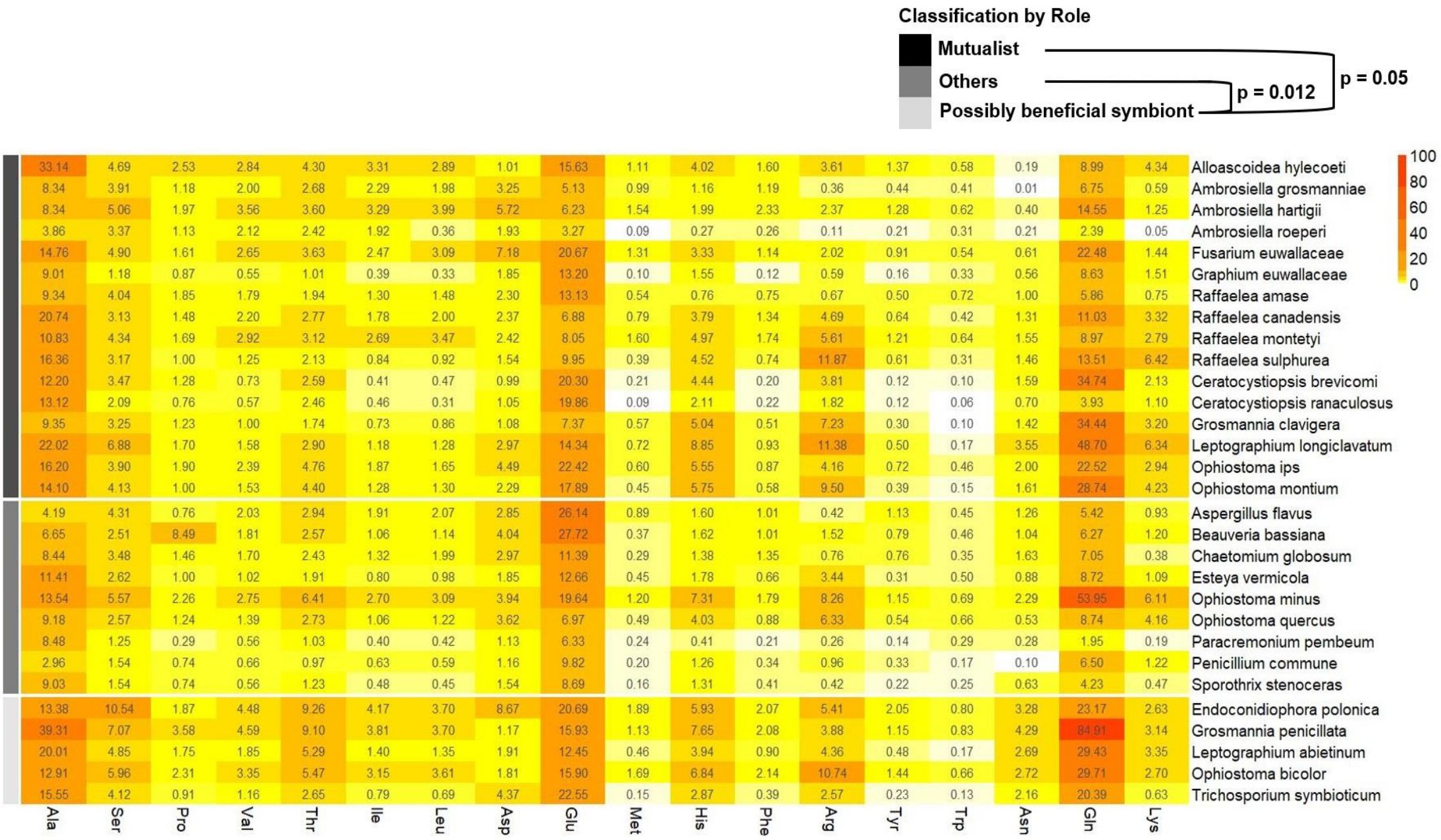
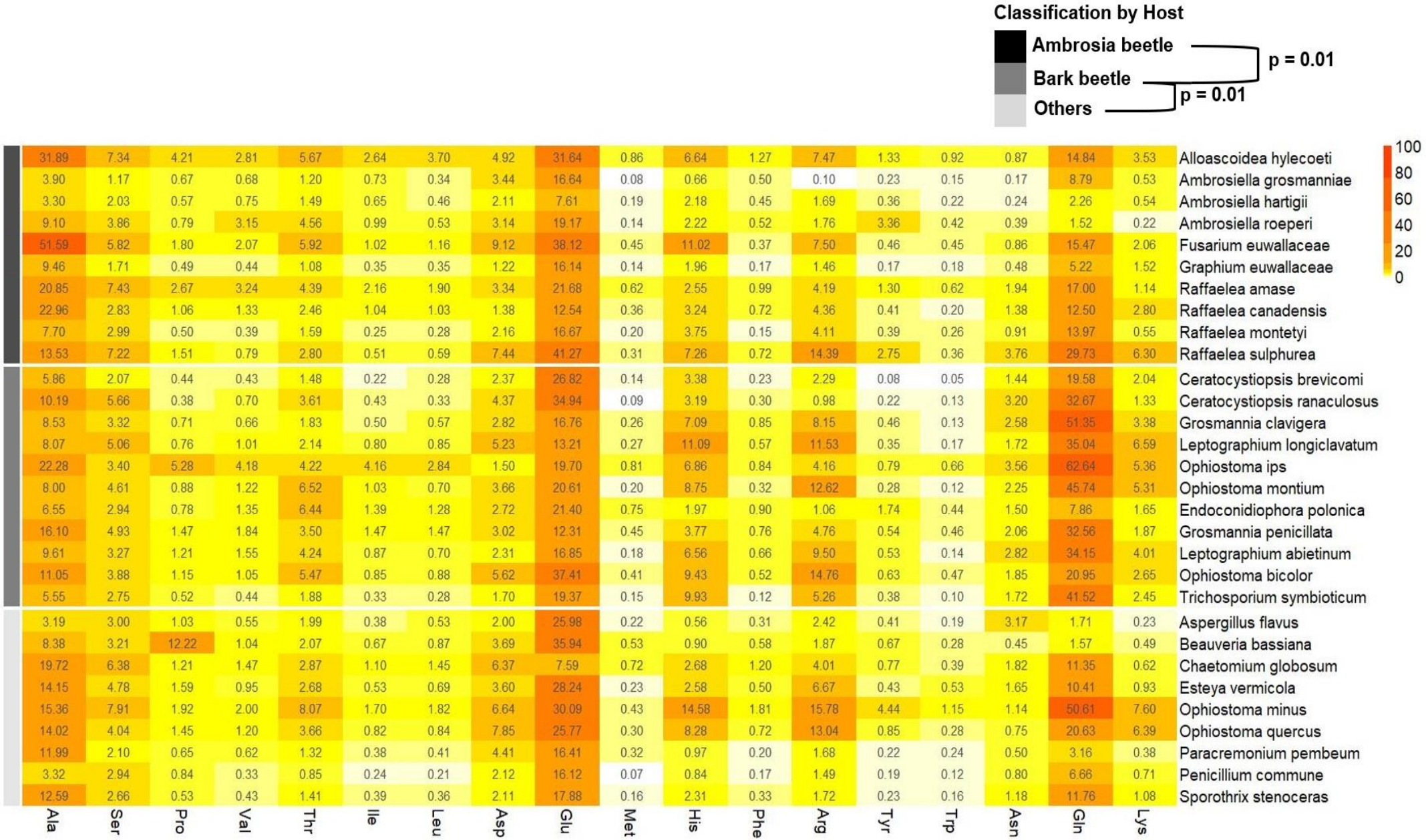


Suppl. Fig. S17



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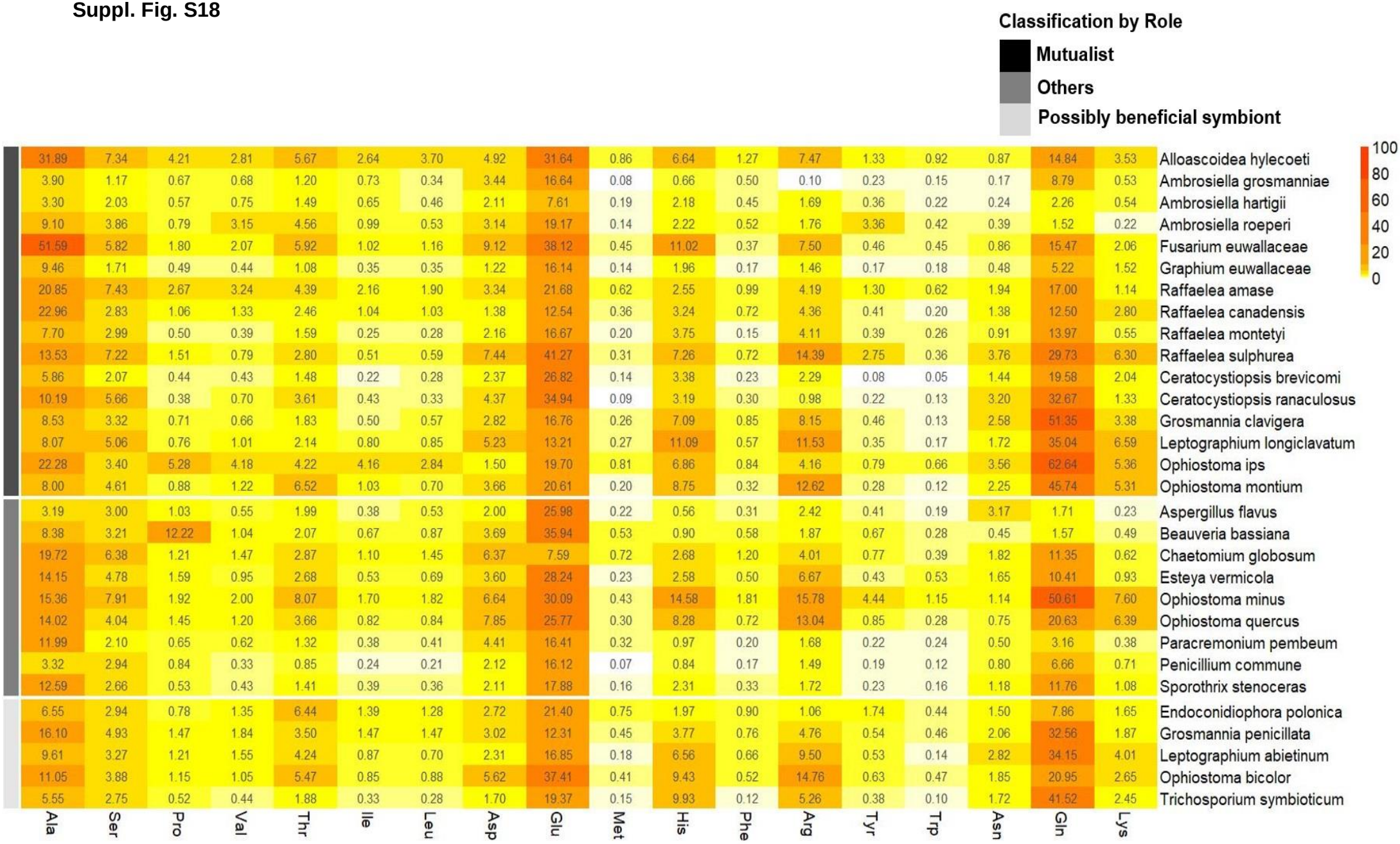

Annex – Supplementary Tables

Suppl. Table S1: Overview of the origin of the 30 fungal isolates used in this study.

Fungal Species	Phylogenetic order	ID	Date of isolation	Isolated by	Locality	Source of isolatation	Sequenced marker	Accession numbers
Raffaelea sulphurea	Ophiostomataceae	P159	2019	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	*Xyleborinus saxesenii*	Beta-tubulin	**MT880108**
Raffaelea montetyi	Ophiostomataceae	P105	2017	Dr. Maria Lurdes Inácio	Portugal	*Platypus cylindrus*	Beta-tubulin	**MT880111**
Raffaelea amase	Ophiostomataceae	CBS116694-C2750-P45	2010	Prof. Dr. Thomas Harrington	Taiwan	*Amasa concitatus*	Beta-tubulin	**MT880112**
Esteya vermicola	Ophiostomataceae	CBS156.82-P167	1999	Liou JY, Shih JY & Tzean; ref.2	Japan	Pinus – dried plant	Beta-tubulin	**MT880110**
Raffaelea canadensis	Ophiostomataceae	P23	2019	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	*Xyleborinus saxesenii*	Beta-tubulin	**MT880109**
Ophiostoma bicolor	Ophiostomataceae	P22	2004	Unknown	Akershus, Norway	*Ips typographus*	LSU	**MT159431**
Sporothrix stenoceras	Ophiostomataceae	P17	2012	Prof. Dr. Peter Biedermann	Bern, Switzerland	None	LSU	**MT252031**
Ophiostoma quercus	Ophiostomataceae	P82	2017	Dr. Maria Lurdes Inácio	Portugal	*Platypus* spp.	LSU	**MT252033**
Trichosporium symbioticum	Ophiostomataceae	FSVo53a-P325	12.08.2018	Prof. Dr. Diana Six	Flathead Lake, Lake County, Montana	*Scolytus ventralis*	LSU	**MT252044**
Ophiostoma ips	Ophiostomataceae	19311-P187	Unknown	Unknown	Unknown	*Ips pini*	LSU	**MT252040**
Ophiostoma minus	Ophiostomataceae	P161	2018	Prof. Dr. Rhichard Hofstetter	Arizona, USA	*Dendroctonus frontalis*	LSU	**MT252036**
Ophiostoma montium	Ophiostomataceae	1892-P316	Aug 10	Prof. Dr. Diana Six	Vipond Park, Pioneer Mtns, Montana	*Dendroctonus ponderosae*	LSU	**MT252042**
Grosmannia penicillata	Ophiostomataceae	2-P189	2006	Unknown	Kronoberg county, Sweden	*Ips typographus*	LSU	**MT159434**

Leptographium abietinum	Ophiostomataceae	1124-P325	July 2002	Prof. Dr. Diana Six	North Lake, Routt National Forest, CA, USA	*Dendroctonus rufipennis*	LSU	**MT252045**
Grosmannia clavigera	Ophiostomataceae	657-P324	June 1995	Prof. Dr. Diana Six	Monitor Pass, Sierra Nevada Mtns, California	*Dendroctonus ponderosae*	LSU	**MT252043**
Leptographium longiclavatum	Ophiostomataceae	D1	Unknown	Unknown	Unknown	*Dendroctonus ponderosae*	ITS	**MT875437**
Ceratocystiopsis brevicomis	Ophiostomataceae	LA12-P328	July 2014	Prof. Dr. Diana Six	La Grande, Oregon	*Dendroctonus brevicomis*	LSU	**MT252046**
Ceratocystiopsis ranaculosus	Ophiostomataceae	949-P320	Unknown	Prof. Dr. Diana Six	Missisippi	*Dendroctonus frontalis*	ITS	**MT875423**
Endoconidiophora polonica	Ceratocystidaceae	F5-P191	2013	Unknown	South Karelia, Finland	*Ips typographus*	LSU	**MT159432**
Ambrosiella grosmanniae	Ceratocystidaceae	P174	2018	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	*Xylosandrus germanus*	LSU	**MT252039**
Ambrosiella roeperi	Ceratocystidaceae	P14	Unknown	Prof. Dr. Peter Biedermann	USA	*Xyleborus crassisculus*	LSU	**MT252030**
Ambrosiella hartigii	Ceratocystidaceae	P137	2019	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	*Anisandrus dispar*	LSU	**MT252034**
Graphium euwallaceae	Microascaceae	P168	2018	Zvi Mendel	Israel	*Euwallacea fornicatus*	LSU	**MT252037**
Paracremonium pembeum	Nectriaceae	P169	2018	Zvi Mendel	Israel	*Euwallacea fornicatus*	LSU	**MT252038**
Beauveria bassiana	Cordycipitaceae	P13	2019	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	*Ips typographus*	LSU	**MT159433**
Fusarium euwallaceae	Nectriaceae	P170	2018	Zvi Mendel	Israel	*Euwallacea fornicatus*	LSU	**MT159437**
Alloascoidea hylecoeti	Alloascoideaceae	P10	Unknwon	Prof. Dr. Peter Biedermann	Bern, Switzerland	*Hylecoetus dermestoides*	LSU	**MT252029**
Penicillium commune	Aspergillaceae	P21	2018	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	None	LSU	**MT252032**
Aspergillus flavus	Aspergillaceae	F15-P153	Unknown	Lehenberger M, following protocol in	Jena, Germany	None	LSU	**MT252035**

				ref.1				
Chaetomium globosum	Chaetomiaceae	P7	2018	Lehenberger M, following protocol in ref.1	Würzburg, Bavaria, Germany	Gallery of *Xyleborinus saxesenii*	LSU	**MT252028**

References Supplementary Table 1

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Supplementary Table 2: Overview of all fungal species used for phylogenetic analysis with the specimen identification number (ID) and the corresponding GenBank accession numbers for the LSU marker. * Species from which we sequenced the LSU-region and which we used for the construction of our phylogenetic tree.

Annotation	ID	Accession Number	Associated beetle	References
Saccharomyces sp. "boulardii"	CIFA-FHMD M1	**MN871795.1**	None	Dharmaraj & Sahoo 2019, unpublished
Allascoidea hylecoeti*	P10	**MT252029**	*Hylecoetus dermestoides*	Lehenberger et al. 2020, unpublished
Candida tropicalis	DMKU-RK553	**AB773417.1**	None	Kaewwichian et al. 2012, unpublished
C. albicans	A_DF_S5888	**KY511828.1**	None	Huyben 2017, unpublished
Penicillium javanicum	Ao78	**MF373378.1**	None	Nayak et al. 2017, unpublished
P. paxilli	Ao89	**MF373380.1**	None	Nayak et al. 2017, Unpublished
P. commune*	P21	**MT252032**	None	Lehenberger et al. 2020, unpublished
Aspergillus flavus*	P153	**MT252035**	None	Lehenberger et al. 2020, unpublished
A. niger	BK 01	**HM008328.1**	None	Cuong & Thu 2010, unpublished
Esteya vermicola	CBS 115803	**EU668903.1**	None	1
Raffaelea quercivora	RA2245	**LC069313.1**	*Platypus quercivorus*	2
R. montetyi	PC06.001	**JF909540.1**	*Platypus cylindrus*	Inacio et al. 2011, unpublished
R. quercus-mongolicae	RQ10.230	**KF513154.1**	*Platypus koryoensis*	Park 2013, unpublished
R. sulphurea	C593	**EU177463.1**	*Xyleborinus saxesenii*	3
R. amase	CBS116694	**EU984295.1**	*Amasa concitatus*	4
R. brunnea	MAR50.a	**MN012913.1**	*Monarthrum* spp.	Restrepo et al. 2019, unpublished
R. lauricola	CBS 121567	**MH877762.1**	*Xyleborus glabratus*	5
R. cyclorhipidia	Jiri Hulcr:9569	**LC363551.1**	*Cyclorhipidion ohnoi*	6
R. santoroi	CBS 399.67	**MH870707.1**	*Platypus* sp.	5

R. sulcati	CBS 806.70	**NG_064084.1**	*Gnathotrichus sulcatus*	5
R. subalba	C2401	**EU177443.1**	*Xyleborus glabratus*	3
R. canadensis	CBS 326.70	**MH871450.1**	*Xyleborinus saxesenii*	5
R. tritirachium	CBS 726.69	**MH871169.1**	*Monarthrum mali*	5
R. albimanens	CBS 271.70	**NG_064077.1**	*Platypus externedentatus*	5
R. fusca	CBS 129011	**MH878043.1**	*Xyleborus glabratus*	5
R. ambrosiae	C2225	**EU177453.1**	*Platypus* spp.	3
R. rapaneae	CMW40359	**KT182935.1**	*Lanurgus* sp.	7
Grosmannia cucullata	CBS 218.83	**NG_064129.1**	*Ips* spp.	5
G. olivacea	CBS 138.51	**NG_064003.1**	*Ips typographus*	5
G. clavigera*	657-P324	**MT252043**	*Dendroctonus ponderosae*	Lehenberger et al. 2020, unpublished
G. aurea	CMW667	**DQ294389.1**	*Dendroctonus* spp.	5
G. laricis	CBS 633.94	**NG_064170.1**	*Ips subelongatus/ Dryocoetes baicalicus*	5
G. penicillata*	P189-2	**MT159434**	*Ips* spp.	Lehenberger et al. 2020, unpublished
G. serpens	CMW 00289	**JN940174.1**	Unknown	8
Leptographium pyrinum	CMW3889	**AY544605.1**	*Dendroctonus adjunctus*	9
L. longiclavatum	M002-11-03-04-UL13G22	**GU370300.1**	*Dendroctonus ponderosae*	10
L. procerum	CBS 128844	**MH876524.1**	*Dendroctonus* spp.	5
L. lundbergii	CBS 128843	**MH878023.1**	*Ips* spp.	5
L. abietinum*	1124-P326	**MT252045**	*Dendroctonus rufipennis*	Lehenberger et al. 2020, unpublished
L. castellanum	CBS 128697	**MH876514.1**	Unknown	5
Ceratocystiopsis minuta-bicolor	CBS 635.66	**MH870571.1**	Unknown	5
C. manitobensis	UM237	**EU913674.1**	Unknown	11
C. brevicomis*	LA12-P328	**MT252046**	*Dendroctonus brevicomis*	Lehenberger et al. 2020, unpublished

C. ranaculosus	CBS 216.88	**EU913673.1**	*Dendroctonus frontalis*	11
Sporothrix stenoceras*	P17	**MT252031**	None	Lehenberger et al. 2020, unpublished
S. splendens	CBS 557.92	**NG_064164.1**	None	5
S. schenckii	CBS 359.36	**KX590890.1**	None	12
Trichosporium symbioticum*	FSVo53a-P325	**MT252044**	*Scolytus ventralis*	Lehenberger et al. 2020, unpublished
Ophiostoma picae	CBS 180.69	**MH877705.1**	Unknown	5
O. canum	CBS 133.51	**MH868299.1**	*Tomicus minor*	5
O. quercus*	P82	**MT252033**	*Platypus* spp.	Lehenberger et al. 2020, unpublished
O. minus*	P161	**MT252036**	*Dendroctonus frontalis*	Lehenberger et al. 2020, unpublished
O. macrosporum	CBS 367.53	**MH868780.1**	*Ips acuminatus*	5
O. piliferum	CBS 138.33	**MH866835.1**	Unknown	5
O. bicolor*	P22	**MT159431**	*Ips* spp.	Lehenberger et al. 2020, unpublished
O. ips*	P187	**MT252040**	*Ips pini*	Lehenberger et al. 2020, unpublished
O. montium*	1892-P316	**MT252042**	*Dendroctonus ponderosae*	Lehenberger et al. 2020, unpublished
Chaetomium globosum*	P7	**MT252028**	None	Lehenberger et al. 2020, unpublished
Neurospora crassa	NA	**AY681158.1**	None	13
Ceratocystis manginecans	CBS 121661	**MH874686.1**	None	5
C. obpyriformis	CBS 122511	**MH874746.1**	None	5
Huntiella moniliformis	14053	**MG954243.1**	None	Huang et al. 2018, unpublished
Endoconidiophora polonica*	F5-P191	**MT159432**	*Ips typographus*	Lehenberger et al. 2020, unpublished
E. coerulescens	CBS 137.34	**MH866946.1**	*Ips subelongatus*	5
Phialophoropsis ferruginea	P233	**MT252041**	*Trypodendron lineatum*	Lehenberger et al. 2020, unpublished
Ambrosiella batrae	CBS 139735	**NG_057134.1**	*Anisandrus sayi*	14
A. hartigii*	P137	**MT252034**	*Anisandrus dispar*	Lehenberger et al. 2020, unpublished

A. beaveri	C2749	**KF646765.2**	*Xylosandrus mutilatus*	15
A. grosmanniae*	P174	**MT252039**	*Xylosandrus germanus*	Lehenberger et al. 2020, unpublished
A. roeperi*	P14	**MT252030**	*Xyleborus crassisculus*	Lehenberger et al. 2020, unpublished
Trichoderma reesei	ATCC 56765	**KU729195.1**	None	Suh 2016, unpublished
T. harzianum	CMF029	**MN307954.1**	None	16
Paracremonium binnewijzendii	MFLUCC 16-1276	**MK828236.1**	Unknown	Luo et al. 2019, unpublished
P. pembeum*	P169	**MT252038**	*Euwallacea fornicatus*	Lehenberger et al. 2020, unpublished
Fusarium oxysporum	NA	**MG597030.1**	None	Li 2017, unpublished
F. euwallaceae*	P170	**MT159437**	*Euwallacea fornicatus*	Lehenberger et al. 2020, unpublished
F. solani	CBS490.63	**AY097316.1**	None	17
Metarhizium brunneum	CBS 316.51	**MH868397.1**	None	5
Beauveria bassiana*	P13	**MT159433**	None	Lehenberger et al. 2020, unpublished
Graphium euwallaceae*	P168	**MT252037**	*Euwallacea fornicatus*	Lehenberger et al. 2020, unpublished
G. fimbriasporum	CMW5605	**KM495388.1**	*Ips* spp.	18

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Suppl. Table S3: Overview of examined time-points and replicates for all examined fungal species separated by the investigation of volatiles and amino acids.

Fungi	Investigation of:	Examined time-point [d]	Replicates [N]
Aspergillus flavus	Volatiles	6; 12	5; 5
Aspergillus flavus	Amino Acids	6; 12	5; 5
Ambrosiella grosmanniae	Volatiles	6; 12	5; 4
Ambrosiella grosmanniae	Amino Acids	6; 12	5; 4
Ambrosiella hartigii	Volatiles	6; 12	5; 5
Ambrosiella hartigii	Amino Acids	6; 12	5; 5
Ascoidea hylecoeti	Volatiles	6; 12	5; 5
Ascoidea hylecoeti	Amino Acids	6; 12	5; 5
Ambrosiella roeperi	Volatiles	6; 12	5; 5
Ambrosiella roeperi	Amino Acids	6; 12	4; 4
Beauveria bassiana	Volatiles	6; 12	5; 5
Beauveria bassiana	Amino Acids	6; 12	5; 5
Ceratocystiopsis brevicomi	Volatiles	6; 12	5; 5
Ceratocystiopsis brevicomi	Amino Acids	6; 12	5; 5
Caetomium globosum	Volatiles	6; 12	5; 5
Caetomium globosum	Amino Acids	6; 12	5; 5
Ceratocystiopsis ranaculosus	Volatiles	6; 12	5; 5
Ceratocystiopsis ranaculosus	Amino Acids	6; 12	5; 5
Endoconidiophora polonica	Volatiles	6; 12	5; 5
Endoconidiophora polonica	Amino Acids	6; 12	5; 5
Esteya vermicola	Volatiles	6; 12	5; 5
Esteya vermicola	Amino Acids	6; 12	5; 5
Fusarium euwallaceae	Volatiles	6; 12	5; 5
Fusarium euwallaceae	Amino Acids	6; 12	5; 5
Grosmannia clavigera	Volatiles	6; 12	5; 5
Grosmannia clavigera	Amino Acids	6; 12	5; 5
Graphium euwallaceae	Volatiles	6; 12	5; 5
Graphium euwallaceae	Amino Acids	6; 12	5; 5
Grosmannia penicillata	Volatiles	6; 12	5; 5
Grosmannia penicillata	Amino Acids	6; 12	5; 5
Leptographium longiclavatum	Volatiles	6; 12	5; 5

Leptographium longiclavatum	Amino Acids	6; 12	5; 5
Ophiostoma bicolor	Volatiles	6; 12	5; 5
Ophiostoma bicolor	Amino Acids	6; 12	5; 4
Ophiostoma ips	Volatiles	6; 12	5; 4
Ophiostoma ips	Amino Acids	6; 12	5; 4
Ophiostoma minus	Volatiles	6; 12	5; 5
Ophiostoma minus	Amino Acids	6; 12	5; 5
Ophiostoma montium	Volatiles	6; 12	5; 5
Ophiostoma montium	Amino Acids	6; 12	5; 5
Ophiostoma quercus	Volatiles	6; 12	5; 5
Ophiostoma quercus	Amino Acids	6; 12	5; 5
Penicillium commune	Volatiles	6; 12	5; 5
Penicillium commune	Amino Acids	6; 12	5; 5
Paracremonium pembeum	Volatiles	6; 12	5; 5
Paracremonium pembeum	Amino Acids	6; 12	5; 5
Raffaelea canadensis	Volatiles	6; 12	5; 5
Raffaelea canadensis	Amino Acids	6; 12	5; 5
Raffaelea amase	Volatiles	6; 12	5; 5
Raffaelea amase	Amino Acids	6; 12	5; 5
Raffaelea montetyi	Volatiles	6; 12	5; 5
Raffaelea montetyi	Amino Acids	6; 12	5; 5
Raffaelea sulphurea	Volatiles	6; 12	5; 5
Raffaelea sulphurea	Amino Acids	6; 12	5; 5
Sporothrix stenoceras	Volatiles	6; 12	5; 5
Sporothrix stenoceras	Amino Acids	6; 12	5; 5
Trichosporium symbioticum	Volatiles	6; 12	5; 5
Trichosporium symbioticum	Amino Acids	6; 12	5; 5
Leptographium abietinum	Volatiles	6; 12	5; 5
Leptographium abietinum	Amino Acids	6; 12	5; 5

General Discussion and Conclusion

Specific filamentous fungi associated with bark and ambrosia beetles are well known for certain beneficial effects towards their beetle hosts (Six 2003; Kirkendall et al., 2015; Biedermann and Vega, 2020). Indeed, some species are playing crucial roles within the beetle-fungus symbiosis and have the potential to facilitate important steps in the beetle's life cycle, such as the colonization of woody substrate and the recognition and/or defense against pathogenic fungi as well as the detoxification of antimicrobial compounds emitted from the host tree, what is highly influencing the survival as well as the reproductive success of the beetles (Wadke et al., 2016; Ranger et al., 2018; Kandasamy et al., 2019; Zhao et al., 2019; Biedermann and Vega 2020). However, several fungi (especially in case of ambrosia beetles) are thought to be even more important, as they enable the beetles to colonize and to survive within certain very resistant woody tissues (e.g. extremely nutrient-poor), and are thus known as necessarily needed key players in maintaining such symbioses (Kirkendall et al., 2015; Biedermann and Vega 2020). Despite the crucial role of symbiotic associated fungi, only some physiological traits of mostly individual or few fungal symbionts associated with bark and ambrosia beetles were examined in detail to date and many questions remain open.

Therefore, I aimed to highlight several important but understudied physiological aspects of convergently evolved mutualistic and/or possibly beneficial symbionts of bark and ambrosia beetles towards their beetle host within my PhD project. Among others, I gained novel insights into several physiological aspects of associated bark and/or ambrosia beetle fungi such as wood-degradation, tolerance/sensibility towards ethanol and the nutritional character of natural beetle galleries as well as the emission of volatile organic compounds.

New aspects within the *Trypodendron* - *Phialophoropsis* mutualism

Fungus-farming ambrosia beetles currently encompass more than 3500 described species worldwide (Hulcr and Stelinski, 2017). Each species is associated with filamentous fungi (besides several other microbes such as bacteria and yeasts) from which specific beneficial fungi, mostly transmitted in highly specialized spore-carrying structures (mycetangia), are overtaking crucial roles and highly influence the survival of their beetle host (Francke-Grosmann, 1956, 1966; Kirkendall et al., 2015; Hulcr and Stelinski, 2017; Biedermann and Vega, 2020). To date, only around 5% of such beneficial fungal symbionts were described and for many ambrosia beetle systems,

the fungal community as well as the presence and morphology of mycetangia are completely unknown (Hulcr and Stelinski, 2017).

Even in case of certain species within the European ambrosia beetle genus *Trypodendron*, which is known to cause economic damages in harvested and stored conifer and broadleaf trees (e.g. Dyer and Chapman, 1965; Schwenke, 1974; Parini and Petercord, 2006), the mutualistic association with *Phialophoropsis* fungi in the Microascales was not safely established when I started my work. The most common European ambrosia beetles from this genus are *T. lineatum*, *T. signatum*, and *T. domesticum*. Indeed, a fourth species exists (*T. leave*) which is widespread but relatively rare and challenging to find (Bussler and Schmidt, 2008). Additionally, mycetangial structures are well known for three of these species (Francke-Grosmann, 1956), but were not investigated for *T. leave* yet.

In terms of the associated fungal mutualist, especially *T. lineatum* is well studied and found to be associated with *P. ferruginea* quite some time ago (Mathiesen-Käärik, 1953; Francke-Grosmann, 1956; Mayers et al., 2015). Although similar fungi were isolated out of *T. domesticum* and *T. signatum* (Nakashima et al., 1992; Mayers et al., 2015), an association could not be safely confirmed due to the low number of isolations and examined beetles. In the first project of my PhD, I confirmed the association of the European ambrosia beetle genus *Trypodendron* with the fungal genus *Phialophoropsis*, and additionally found species-specific differences between the fungal isolates of *T. lineatum* and *T. domesticum*/*T. signatum* (see Chapter 1: Lehenberger et al., 2018). *T. lineatum* was found to be associated with *P. ferruginea*, while *T. signatum* as well as *T. domesticum* are apparently associated with a possible new species in the genus *Phialophoropsis*. Indeed, a recent study confirmed my results that both *T. domesticum* and *T. signatum* are sharing the same fungal symbiont, which was annotated as a new *Phialophoropsis* species (Mayers et al., 2020).

Mycetangial structures have essential roles in the transmission of mutualistic fungi and were examined in detail by Francke-Grosmann (1956) for the genus *Trypodendron*. In my second project, I provided first µCT images of the mycetangium of *T. leave*, which was unknown before (see Chapter 2: Lehenberger et al., 2019). The revealed paired, tubular, pleural-prothoracic mycetangium is typical for *Trypodendron* ambrosia beetles and fits well with the schematic structures provided by Francke-Grosmann (1956), which are all morphologically quite similar.

Novel insights into the symbiosis of bark and ambrosia beetles with beneficial filamentous fungi

The colonization of both bark/phloem (in case of bark beetles) and xylem (in case of ambrosia beetles) of woody plants necessarily led to a highly evolved ecology and several further adaptions of these wood-boring insects (Kirkendall et al., 2015). Otherwise foraging within such challenging lignified substrates would not have been possible. Indeed, within the past 90 – 120 million years (when this wood-boring trait originated), evolutionary processes selected for very specific morphological and ecological strategies to overcome various competitive/defensive conditions within the beetle's life cycle (Kirejtshuk et al., 2009; Jordal et al., 2011; McKenna, 2011; Santos et al., 2011; Haran et al., 2013; Kirkendall et al. 2015). One of the most important adaptions may be the association with microorganisms. The beneficial effects of such associations are well known for bark and ambrosia beetles, but also from several other insects as well as plants (e.g. Six, 2003, 2013; Douglas, 2015; Card et al., 2015; Parnell et al., 2016; Chomicki and Renner, 2017; Ankrah and Douglas, 2018; Biedermann and Vega, 2020), where surviving without symbionts may either be impossible or extremely difficult. Especially within bark and ambrosia beetle systems, certain associated filamentous fungi are known to fulfill crucial functions, as they can serve as a highly nutritional source of food and/or are able to overcome defensive mechanisms of colonized trees and compete with fungal antagonists (e.g. Wadke et al., 2016; Six 2003, 2013; Ranger et al., 2018; Biedermann and Vega, 2020).

Surviving in an extremely nutrient-poor environment

However, the utilization and dependence on fungi as a source of nutrition is not unique to bark and ambrosia beetles, as this trait also evolved within attine ants and termites (Müller et al., 2002). These ants and termites have to provide plant biomass for the cultivation of their fungi, which they actively collect and transfer to their colonies. In contrast, bark and ambrosia beetles are typically not leaving their galleries for almost their entire life cycle (Kirkendall et al., 2015). Thus, they depend on the capability of fungi (and other microbes) to decompose the main carbon sources (crucially needed for all living organisms) of woody tissue, which mainly consists of cellulose, hemicelluloses (such as xylan and mannan), pectin, and lignin (as these compounds are indigestible by insects) (Douglas, 2009). Indeed, this trait is thought to be especially the case in ambrosia beetles, which are colonizing the extremely nutrient poor sapwood of trees, while bark beetles are generally excavating

their galleries in bark and phloem, a richer source of nutrition, where certain fungi may only have supporting effects in terms of nutrition (e.g. Francke-Grosmann, 1967; Norris, 1979; Beaver et al., 1989; Pretzsch et al., 2014; Kirkendall et al. 2015; Ulbricht et al., 2016).

Ambrosia beetle fungi are generally believed to be rather poor wood-decomposers, unable to grow far into the xylem (< 2-3 mm) (Francke-Grosmann, 1966). However, I showed that *Phialophoropsis* fungi, associated with *Trypodendron* ambrosia beetles, are able to express robust amounts of endo-β-1,4-xylanase and endo–β-1,4-glucanase (see Chapter 1: Lehenberger et al., 2018). This ability may allow the fungi to degrade and utilize particularly cellulose and xylan as a carbon source. Interestingly, fungi of the genus *Phialophoropsis* are among the very few species (e.g. *Fusarium euwallaceae*), which are thought or known to forage deeper within the xylem (Francke-Grosmann, 1966; Eskalen et al., 2013). Except De Fine Licht and Biedermann (2012), which examined whole breeding systems of *X. saxesenii* containing several other microbes, representative studies are currently missing. It remains unclear how widespread the importance of wood-degrading enzymes is within ambrosia beetle fungi as well as within currently untested bark beetle fungi or if other microbes are involved. Possibly, several other mutualistic fungi are receiving only low amounts (or none) of carbon out of cellulose and hemicelluloses, and instead are focusing on other woody polysaccharides such as pectin as already suggested by Francke-Grosmann (1966).

In case of ambrosia beetle fungi, it would be also possible that at least some carbon is coming from ethanol, which is emitted by the colonized tree as well as by several bark and ambrosia beetle fungi (see Chapter 3: Lehenberger et al., 2021 and Chapter 5). In my third project, I could show that mutualistic ambrosia beetle fungi benefit from lower ethanol concentrations as they produced higher amounts of biomass than without ethanol. Indeed, this may indicate the ability to metabolize and thus to utilize ethanol as a carbon source (see also Ranger et al., 2018), a trait also known for other fungi (Strijbis and Distel, 2010). However, it might be also possible that ethanol is acting as a growth inducer as shown within other fungal species (e.g. Sortkjaer and Allermann, 1972; Meza et al., 2007). Further physiological and genomic studies would be needed to find out how and through which pathways beetles are receiving their carbon.

In my fourth project I showed that natural galleries of three examined ambrosia beetles are highly enriched with several elements (especially with nitrogen, sulfur, phosphorus, potassium, and magnesium) in contrast to the immediate surrounding xylem and completely uncolonized xylem (see Chapter 4). This raises the questions, who concentrated these elements within the galleries and how and from where they are coming as xylem generally contains only very low concentrations (Fengel and Wegener, 1989; Filipiak et al., 2016). Except nitrogen, which may be provided by N-fixing bacteria as described within bark beetle systems, see Peklo and Satava, 1949 and Bridges, 1981), none of the other elements are fixed from the atmosphere and galleries are isolated from the external environment. Certain microbes might generally be good candidates for translocation and enrichment with nutrients. However, bacteria and yeasts are generally unicellular organisms, so they cannot grow into wood and translocate elements from xylem to the galleries, what excludes them from the list of possible candidates. Mutualistic ambrosia beetle fungi would be good candidates for translocating and concentrating elements from the immediate surrounding xylem within the beetle's galleries as recently shown within a *Dendroctonus* bark beetle system (Six and Elser, 2019, 2020). As ambrosia beetle fungi are known to be of high nutritional value (Batra, 1967), they may have evolved certain mechanisms to gather important elements out of their nutrient-poor environment. Indeed, through my studies I could partly confirm this nutritional character based on the absolute content of 18 free amino acids within the examined fungal biomass (see Chapter 5). Here, both mutualistic bark and ambrosia beetle fungi as well as possibly beneficial symbionts were generally showing high amino acid concentrations. The identified 18 free amino acids were also present in non-mutualistic and free-living fungi but mostly with lower concentrations. Regarding the provision of elements within the beetles galleries, I therefore suggest that mutualistic ambrosia beetle fungi are translocating and concentrating certain elements out of the immediate surrounding xylem (< 2 mm), which is fed by the elemental pipeline of the colonized tree between roots and leaves (and vice versa) (see Matyssek et al., 2010) to the beetles galleries. To confirm this assumption, further experiments including the utilization of stable isotopes are needed and would help to understand underlying processes.

Beetle associated fungi emit several volatile organic compounds (VOC)

Bark and ambrosia beetles are generally known to use chemical/mechanic cues for the selection of their host tree and mate location, such as taste, olfaction and

possibly also sound (e.g. Rudinsky and Michael, 1974; Ryker, 1984; Raffa et al., 2015; Hofstetter et al., 2019). However, it has been a long-standing question, how and if beetles can distinguish among certain fungi within their galleries to locate their beneficial fungi as well as to identify possible antagonistic fungi. Indeed, the ambrosia beetle *Xyleborinus saxesenii* was observed to actively remove fungal antagonists and thus, to prevent these competitive fungi from spreading (Biedermann et al., 2013; Nuotclà et al., 2019), but how the beetles are informed about the presence of such fungi remains mostly unknown. Nowadays, it is known for some beetle-fungus symbioses, that olfaction plays a role. As shown within past studies (Hulcr et al., 2011; Kandasamy et al., 2016, 2019 but see also Chapter 5), VOCs can have attractive or repellent (or none) effects towards beetles. Indeed, fungal VOCs can generally fulfill various functions towards plants, animals, and other fungi as summarized by Cale et al. (2016). In case of bark beetles, especially the study by Kandasamy et al. (2019) highlights crucial functions of fungal volatiles in maintaining the symbiosis of *Ips typographus* with its fungal symbionts (see Introduction and Chapter 5). My studies showed that the majority of examined mutualistic and possibly beneficial symbionts of bark and ambrosia beetles are emitting volatile organic compounds mainly classified into aliphatic and aromatic alcohols and esters (similar to Kandasamy et al., 2019 but see also summary in Kandasamy et al. 2016 for already investigated bark beetle fungi). In contrast, antagonistic and free-living fungi emitted a lower number as well as lower amounts of volatiles. This finding is fascinating as it indicates a crucial role of fungal volatiles within several and widespread bark and ambrosia beetle systems and has the potential to be one of the main drivers of the symbiosis. Additionally, the study on fungal volatile organic compounds associated with bark and ambrosia beetles can serve as the base for developing olfaction-based management strategies for several beetle species.

Novel insights into the suppression of antagonistic fungi in ambrosia beetle systems

All bark and ambrosia beetles generally have to compete with ubiquitous antagonistic fungi such as *Chaetomium globosum* (in case of ambrosia beetles) and *Trichoderma harzianum* (in case of bark beetles) as well as with entomo-pathogenic fungi such as *Beauveria bassiana*, which can either overgrow beneficial fungi and/or kill the beetles and their larvae (Jassim et al., 1990; Shakeri and Foster, 2007; Castrillo et al., 2011; Bezos et al., 2018; Nuotclà et al., 2019). If such aggressive and fast-growing fungi

are becoming more dominant with the beetle's galleries, the risk that beetles and larvae are either dying or are at least performing poorly is quite high. To date, it is unclear for the majority of bark and ambrosia beetles, how (and if in case of several bark beetles) they protect their fungal cultivars against antagonistic "weedy" fungi. The primitively eusocial ambrosia beetles *Xyleborinus saxesenii* is well known for its cleaning behavior, where especially larvae were observed to clean colony members as well as the walls in their galleries, what minimizes the abundance of certain antagonistic fungi (Biedermann et al., 2013). It was also observed, that as soon as ambrosia beetles are leaving the natal nest, antagonistic fungi are overgrowing the ambrosia beetle cultivars (Hubbard, 1897; Francke-Grosmann, 1956). So apparently, the beetles themselves have a great influence on the composition of their fungal gardens.

A recent study showed that the beetles are not solely suppressing antagonistic fungi (Ranger et al., 2018). Here, the authors found, that ethanol, which is generally emitted from host trees and indicates susceptible substrate (Graham, 1968; Kimmerer and Kozlowski, 1982; Kimmerer and Stringer, 1988; Kimmerer et al., 1991; Kühnholz et al., 2001; Ranger et al., 2015; Kelsey et al., 2014) has not only important functions in terms of host finding for the beetles. It also serves as the base for an environmental screening, where ethanol tolerant species are benefitted, while species sensitive to ethanol are inhibited. Within my studies, I confirmed these previous findings (ethanol tolerance in ambrosia beetle fungi) for a broader range of former unstudied ambrosia beetle fungal species (see Chapter 3: Lehenberger et al., 2021) suggesting that ethanol tolerance might be a widespread trait within several ambrosia beetle systems. Further, examined fungal antagonists where highly inhibited by ethanol, what indicates the mechanism of environmental screening (ambrosia beetle fungi benefit, while antagonists are suppressed).

However, the majority of tested bark beetle fungi were highly inhibited through even low concentrations of ethanol. This is especially surprising as ethanol also naturally occurs within the phloem of trees (see above) and is additionally also produced by both bark and ambrosia beetle fungi (see Chapter 5) as well as by several other species (for details see Chapter 3). Here, examined bark beetle fungi apparently emitted even higher concentrations of ethanol compared to ambrosia beetle fungi (as I used a semi-quantitative pattern here, this needs to be discussed with care). However, ethanol is a general metabolic product in fungi, deriving from fermentation

processes (degradation of sugars, what is needed to gain carbon) under oxygen limited conditions and serves as the base for the production of ethyl acetate (Skory et al., 1997; Strijbis and Distel, 2010; Wang et al., 2016; Holt et al., 2019). Ethanol (as well as acetate) can further be used as the base for the production of higher order carbohydrates (see Strijbis and Distel, 2010). Therefore, it might be possible that the studied bark beetle fungi are more efficient in sugar degradation (more free sugars such as glucose available within phloem compared to xylem, see Matyssek et al., 2010) and may additionally utilize more ethanol for other processes compared to ambrosia beetle fungi. Indeed, the emitted amount of ethanol was much lower compared to the concentrations I applied to test for ethanol tolerance/sensitivity (see Chapter 3 and Chapter 5). As bark beetles are colonizing phloem, it might additionally be easier for ethanol to evaporate (what would decrease the ethanol concentrations within galleries) compared to evaporation within the xylem. This might explain why there is no urgent need to detoxify high ethanol concentrations within bark beetle fungi but indicates the need within ambrosia beetle fungi. It is also worth pointing out that the effect of ethanol as a volatile organic compound has not been tested on bark and ambrosia beetle fungi to date.

Bark and ambrosia beetles are emitting various volatile organic compounds (see Chapter 5). Interestingly, the majority of mutualistic fungi as well as of possibly beneficial mutualists emitted several compounds, which are known to have antifungal capabilities (for details see Chapter 5). Possibly, the emission of certain volatiles can have beneficial effects towards bark and ambrosia beetle fungi. In case fungal antagonists would be inhibited by specific released volatiles, while the mutualistic or possibly beneficial symbiont are not affected, this would be fascinating as it indicates the presence of other/further mechanisms for environmental screening. However, inhibitory effects of certain fungal volatiles towards competitive fungi were already observed within other fungus – fungus interactions (see Amin et al., 2010 and Nishino et al., 2013), but remain unknown for bark and ambrosia beetle systems. Here, experiments excluding physical contact between fungi and tested volatiles as well as between two fungi could show if certain fungi are inhibited or unaffected by VOCs emitted from bark and ambrosia beetle fungi (similar to Cale et al., 2016).

Summary / Zusammenfassung

Within my PhD project I gained several novel insights into the poorly investigated symbiotic world of fungus farming ambrosia beetles and their bark beetle ancestors, where I especially focused on physiological interactions and capabilities of associated fungal symbionts. Here, (**i**) I could confirm the association of mutualistic *Phialophoropsis* fungi with the ambrosia beetle genus *Trypodendron* and found hints for a possible new *Phialophoropsis* species in *T. signatum* and *T. domesticum*. Moreover, I could show that mutualistic fungi of *Trypodendron* ambrosia beetles are able to decompose major woody polysaccharides such as cellulose and xylan. Additionally, (**ii**) I provided the first images using micro-computed tomography (µCT) of the formerly unknown structure of the mycetangium of *Trypodendron leave*. (**iii**) I could confirm a general tolerance towards ethanol in mutualistic ambrosia beetle fungi, while antagonistic fungi as well as most examined fungal bark beetle associates (e.g. possibly tree-defense detoxifying species) were highly sensitive to even low concentrations of ethanol. Further, (**iv**) I found that natural galleries of ambrosia beetles are highly enriched with several biologically important elements (such as N, P, S, K, Mg) compared to the surrounding woody tissue and suggest that mutualistic fungi are translocating and concentrating elements from the immediate surrounding xylem to the beetles galleries. Furthermore, (**v**) I could show that various fungi associated with bark and ambrosia beetles (mutualists, possibly beneficial symbionts) are emitting several volatile organic compounds mostly within aliphatic and aromatic alcohols and esters, while non-mutualistic and free living species were generally emitting a lower number and amount of volatiles. Finally, especially bark and ambrosia beetle fungi were found to incorporate several amino acids, from which some are especially important for the production of certain volatile organic compounds. Amino acid content also indicated a higher nutritional value for certain species. Here, I propose that especially volatile organic compounds are widespread key players in maintaining various symbioses between fungi and beetles, as already proven by a recent study on the bark beetle *Ips typographus* (as well as for some other bark beetle-fungus symbioses, see summary in Kandasamy et al. 2016) and also suggested for ambrosia beetles.

Im Rahmen meiner Promotion konnte ich einige neue Einblicke in die symbiotische Welt von Pilz-züchtenden Ambrosiakäfern sowie ihren direkten Vorfahren den Borkenkäfern, mit speziellen Fokus auf physiologische Interaktionen und Besonderheiten der assoziierten Pilzen, erlangen. Hier konnte ich (**i**) die Assoziation der europäischen *Trypodendron* Arten mit mutualistischen Pilzen in der Gattung *Phialophoropsis* generell bestätigen und fand des weiteren Hinweise auf eine vermutlich neue *Phialophoropsis* Art in den beiden Ambrosiakäfern *T. domesticum* und *T. signatum*. Außerdem konnte ich zeigen, dass mutualistische Pilze, welche mit Ambrosiakäfern der Gattung *Trypodendron* assoziiert sind, wesentliche Polysaccharide im Holz (Cellulose und Xylan) abbauen können. Zusätzlich war es mir möglich, (**ii**) erstmals die Pilzsporen übertragende Struktur „Mycetangium“ des Ambrosiakäfers *Trypodendron laeve* zu untersuchen und durch den Einsatz von Mikro-Computertomographie (µCT) somit die ersten detaillierten Aufnahmen bereit zu stellen. Außerdem bestätigten und ergänzten meine Studien, (**iii**) das Ethanol-Toleranz unter Ambrosiakäfer-Pilzen eine wohl sehr weit verbreitete Besonderheit ist, wohingegen für die Käfer bzw. für die Ambrosiapilze schädliche Pilze stark durch schon geringe Ethanol-Konzentrationen gehemmt werden. Interessanterweise wurden auch nahezu alle mit Borkenkäfern assoziierten Pilze stark durch Ethanol gehemmt. Des Weiteren konnte ich zeigen, (**iv**) das natürliche Brutsysteme von Ambrosiakäfern, im Gegensatz zu unbesiedeltem Splintholz, sehr stark mit verschiedenen essentiellen Elementen (wie etwa N, P, S, K und Mg) angereichert sind. Sehr wahrscheinlich ist es den Pilzsymbionten möglich, Elemente aus unmittelbar umliegendem Splintholz abzuziehen und diese in den Brutsystemen der Käfer entsprechend anzureichern. Außerdem konnte ich zeigen, (**v**) das nahezu alle untersuchten Borken- und Ambrosiakäfer-Pilze (Mutualisten sowie möglicherweise begünstigende Symbionten) eine Vielzahl an flüchtigen Inhaltsstoffen produzieren, welche sich hier vor allem in die Gruppe der aliphatischen und aromatischen Alkohole und Ester eingliedern. Nicht mutualistische Pilze sowie freilebende Arten produzierten im Vergleich eine geringere Anzahl an unterschiedlichen flüchtigen Inhaltsstoffen und emittierten zumeist geringere Mengen davon. Schließlich konnte ich zeigen, dass vor allem Pilze welche mit Borken- und Ambrosiakäfern assoziiert sind, größere Mengen an verschiedenen Aminosäuren in ihrer Biomasse einbauen, von welchen einige besonders wichtig sind um bestimmte flüchtige Inhaltsstoffe zu bilden. Dazu kann durch den Anteil der gefundenen Aminosäuren in der Pilzbiomasse auf den nährstoffreichen Charakter der Pilze geschlossen werden.

Diese flüchtigen Inhaltsstoffe haben mit sehr hoher Wahrscheinlichkeit eine essenzielle Rolle innerhalb einer Vielzahl an Käfer-Pilz Symbiosen und sind vermutlich maßgeblich an dem Erfolg und Bestand solcher Symbiosen beteiligt. Dies wurde bereits in jüngster Vergangenheit bei dem Borkenkäfer *Ips typographus* mit seinen assoziierten Pilzen gezeigt (aber auch bei einigen weiteren Borkenkäfern, siehe Zusammenfassung von Kandasamy et al., 2016), während es bei Ambrosiakäfern bisher nur vermutet wurde.

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Statement of individual author contributions and of legal second publication rights

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1. **Publication:** Lehenberger M., Biedermann, P. H. W., & J. Benz, P. (2018). Molecular identification and enzymatic profiling of *Trypodendron* (Curculionidae: Xyloterini) ambrosia beetle-associated fungi of the genus *Phialophoropsis* (Microascales: Ceratocystidaceae). Fungal Ecology. 38: 89 – 97. doi.org/10.1016/j.funeco.2018.07.010

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3	ML	BPHW			
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Explanations (if applicable):

I also confirm my primary supervisor's acceptance.

Maximilian Lehenberger

Doctoral Researcher's Name Date Place Signature

Affidavit

I hereby confirm that my thesis entitled Ecology and Evolution of symbiotic microbial communities in fungus farming ambrosia beetles is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation Ökologie und Evolution von symbiotischen mikrobiellenen Gemeinschaften in Pilz-züchtenden Ambrosia Käfern eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift