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Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae, Scolytinae)

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Abstract

Insect fungus gardens consist of a community of interacting microbes that can have either beneficial or detrimental effects to the farmers. In contrast to fungus-farming ants and termites, the fungal communities of ambrosia beetles and the effects of particular fungal species on the farmers are largely unknown. Here we used a laboratory rearing technique for studying the filamentous fungal garden community of the ambrosia beetle, *Xyleborinus saxesenii*, which cultivates fungi in tunnels excavated within dead trees. *Raffaelea sulfurea* and *Fusicolla acetilerea* were transmitted in spore-carrying organs by gallery founding females and established first in new gardens. *R. sulfurea* had positive effects on egg-laying and larval numbers. Over time, four other fungal species emerged in the gardens. Prevalence of one of them, *Paecilomyces variotii,* correlated negatively with larval numbers and can be harmful to adults by forming biofilms on their bodies. It also comprised the main portion of garden material removed from galleries by adults. Our data suggests that two mutualistic, several commensalistic and one to two pathogenic filamentous fungi are associated with *X. saxesenii*. Fungal diversity in gardens of ambrosia beetles appears to be much lower than in gardens of fungus-culturing ants, which seems to result from essential differences in substrates and behaviours.

Introduction

Mycophagy by insects has evolved in several lineages including springtails, flies, moths, wood wasps, termites, ants and beetles (Wheeler and Blackwell 1984; Martin 1987; Wilding et al. 1989). Among these, only attine ants, macrotermitine termites and curculionid ambrosia beetles evolved advanced fungus agriculture (Mueller et al. 2005). This involves (1) obligate nutritional dependence on fungal food for adults and their brood, (2) translocation of their fungal crops by spore- or propagule-carrying organs within nests and when founding new nests, and (3) cultivation and management of the fungal crops (i.e., continuous monitoring, management and protection, weeding, control of alien microbes). As the latter can be easier managed by a group of individuals partitioning the labour, advanced fungus agriculture is often associated either with a subsocial (most ambrosia beetles) or eusocial life strategy (all farming ants and termites, one ambrosia beetle; Mueller et al., 2005).

Fungus agriculture has been well studied in the fungus-farming ants and termites, but is little understood in ambrosia beetles. These beetles dwell in the wood of (usually) recently dead or weakened trees, where they construct tunnel systems (galleries) upon the walls of which they nurture ambrosia gardens. Ambrosia gardens consist of fungi the beetles carry into trees in their guts or, more commonly, in specialized structures called mycetangia or mycangia (Francke-Grosmann

1956; 1975). This vertical transmission of ambrosia fungi from the beetle`s natal galleries to newly founded nests can support co-evolution between fungi and beetles and a species-specific association between partners (Six 2003). For many species, healthy fungus gardens are dominated by mutualistic ambrosia fungi of the genera *Raffaelea* and *Ambrosiella* (Ascomycota) (Harrington et al. 2010). These species usually show an ambrosial growth form within the gardens forming nutrient-rich fruiting structures (e.g. conidiospores) that are grazed by adult beetles and their offspring. Gardens also contain a complex of other filamentous fungi, yeasts and bacteria (e.g., Haanstad and Norris 1985), which are often transmitted by spores sticking to the body of founding females (Francke-Grosmann 1967). The effects of these microbes are largely unknown, but antibiotic roles of some bacteria similar to those found in scolytine bark beetles (Adams et al. 2008; Scott et al. 2008) may occur.

The full community of associated fungi of only three of the approx. 3500 ambrosia beetle species worldwide have yet been investigated (Kajimura and Hijii 1992; Harrington and Fraedrich 2010; Endoh et al. 2011), and the fungal dynamics in mycangia and galleries have been studied only in one ambrosia beetle, *Xylosandrus mutilatus* (Kajimura and Hijii 1992). Management of fungal associates by ambrosia beetles within their tunnels has not yet been studied, but there are hints that both adults and larvae are able to influence the composition of the fungal community of their ambrosia gardens (e.g. Beaver 1989, Cardoza et al. 2006, Biedermann and Taborsky 2011); this might happen in a similar manner as in fungus-farming ants (Mueller et al. 2001). Adult ambrosia beetles are particularly attracted to their primary ambrosia fungus and repelled by fungal pathogens (Hulcr et al. 2011). An observational study of ambrosia beetle behaviours within their nests revealed that adults and larvae interact closely with their gardens (Biedermann and Taborsky 2011). Larvae were observed to cooperate in various duties, which is exceptional for holometabolous insects (larval workers are only known from termites (e.g. Korb 2008)); *X. saxesenii* larvae enlarge the gallery by digging and thereby create space for the fungi to spread, they fertilize fungi with their excretions, clean colony members and gallery walls which prevents the spreading of mold, and participate in the removal of waste from the tunnel system (Biedermann and Taborsky 2011). Adults were observed to block tunnels, thus potentially regulating the microclimate of the gardens (Kirkendall et al. 1997), to graze their gardens which apparently induces ambrosial growth (French and Roeper 1972) and potentially also affects species composition (Biedermann and Taborsky 2011), and to deposit and remove waste from the gallery (Biedermann and Taborsky 2011). Ambrosia fungi have been shown to dominate gardens in recently founded galleries and newly built tunnel systems, and to decrease in abundance relative to invading weed fungi at the end of gallery life when beetles leave the nest, and in old parts of the tunnel system (e.g. Fischer 1954; Kajimura and Hijii 1992). The relative prevalence of various fungi may positively or negatively affect the brood. For example, fungal associates of

Xyleborus ferrugineus (Fabricius) vary considerably in sterol, lipid and amino acid content, and thus in their nutritional quality for the developing brood (Kok and Norris 1972a, b; 1973).

Here we report a comprehensive survey of the filamentous fungi closely associated with the ambrosia beetle, *Xyleborinus saxesenii* Ratzeburg, and their dynamics in relation to the life history of this beetle, which hints on the functional relationships between specific fungi and their beetle host. In addition, we report which fungal associates of *X. saxesenii* are carried in the mycetangia and guts of females during their dispersal flight. Based on previous studies we expected to find *Raffaelea sulfurea* (L.R. Batra) T.C. Harr. (previously *Ambrosiella sulfurea*; Harrington et al. 2010) as the primary symbiont (Francke-Grosmann 1956; 1975; Batra 1967; Roeper et al. 1980; Roeper and French 1981), but hitherto the identity of other fungal associates has been unknown. To follow the dynamics of the fungal community within the beetle gardens and their effect on the brood, we sampled garden material over the entire developmental period of a brood and recorded brood numbers and offspring development. This was possible through the use of a laboratory rearing technique that allowed us to observe the beetles within their galleries (Biedermann et al. 2009). Furthermore, we identified (1) fungi removed from the galleries by adults (to the dumps; i.e. the material disposed of out of the entrance tunnel), and (2) detrimental fungi growing on the bodies of beetles. Finally, we discuss our results in comparison to fungal communities found within gardens of fungus-growing ants.

Materials & Methods

Study species

Xyleborinus saxesenii is one of the most common ambrosia beetles in temperate zones worldwide. Originally native to Eurasia, over the last 200 years it has been introduced into parts of Africa, Oceania, as well as South and North America (for the actual distribution see http://xyleborini.tamu.edu/public/site/scolytinae/home). The species is still spreading, facilitated by the shipment of timber products around the world, and by characteristics of its own biology. *X. saxesenii* shows little host tree preference and a mating system in which sib-mating between haploid brothers and diploid sisters in their natal nest is the rule (comparable to *Xylosandrus germanus*; Peer and Taborsky 2004, 2005). Therefore, the translocation of a single already mated female may be sufficient for the successful establishment of a new population.

Galleries of *X. saxesenii* are founded by single females which dig a vertical entrance tunnel extending a few centimetres into a tree trunk. They inoculate gallery walls with fungi, lay eggs when fungal gardens have established, and later care for the developing brood. The larvae feed on fungus-infested wood and in this way gradually enlarge the tunnel to a flat brood chamber (Roeper 1995). This xylomycetophagous feeding is typical for larvae in the genus *Xyleborinus* and likely serves to

reduce kin competition, as it increases the space for ambrosia gardens to grow and improves the breakdown of wood by enzymes (De Fine Licht and Biedermann 2012). Wood passes through the guts of larvae without being digested, but in the process it is finely ground into a form readily utilized by the fungi. Such woody frass is partly spread on the ambrosia garden microbes, which probably recycle and fully breakdown this material (Biedermann and Taborsky 2011).

Overlapping generations are typical in *X. saxesenii* nests, because adult females delay dispersal after maturation and fertilization by a brother (Peer and Taborsky 2007; Biedermann et al. 2011). During this time they engage in brood and fungus care, thereby increasing gallery productivity (Peer and Taborsky 2007; Biedermann and Taborsky 2011). Additionally, fungal gardens benefit from the recycling of their excretions (Abrahamson and Norris 1970). About 20% of daughters also reproduce in their natal nest (Biedermann 2007; Biedermann et al. 2011).

Beetle collection and laboratory breeding

About 100 *X. saxesenii* females were caught live in Lindgren funnel traps baited with ethanol in Pineville, LA, USA (38m asl; 31°20', 92°24') during the summer of 2007. Collection cups were filled with damp sterile filter paper and emptied twice daily to avoid microbial contamination of the beetles (Benjamin et al. 2004). In the lab, we surface-sterilized the beetles by rinsing them twice for a few seconds, first with 70% ethanol and afterwards with deionised water. This treatment does not harm the fungal spores of the cultivar within the mycetangium, but reduces external contamination by eliminating some of the spore-load sticking to the body surface of the beetles (e.g. molds). It is necessary for laboratory breeding of ambrosia beetles, because these contaminants establish more easily in standardized artificial medium than under natural conditions (Biedermann et al. 2009), where beetles largely surface-sterilize themselves boring through bark rich in fungitoxins and other antibiotic substances (Berryman 1989). Surface-sterilization – both in the laboratory and in the field – is incomplete, however, because specific contaminants eventually take-over old galleries (Kajimura and Hijii 1992), which are transmitted initially as sticky spores in pits of the exoskeleton.

Apart from thirteen females used for fungal isolations (see below), the collected females were placed singly on an agar-sawdust-based rearing medium in glass tubes (for details on this technique and ingredients of the *modified medium* see Biedermann et al. (2009)). Tubes were closed with plastic caps, stored vertically, and wrapped in paper in a way that allowed light to penetrate the tube only from the top. This way beetles frequently bored tunnels next to walls of the glass tube, allowing observations of brood development when the paper was removed (Biedermann 2007; Biedermann et al. 2011). Tubes were kept at 23°C.

Fungus isolations from adult females captured during dispersal flight

After surface-sterilization, we aseptically dissected mycetangia from 13 adult females using fine tweezers under a microscope (6.4× – 40× magnification). The mycetangium in *X. saxesenii* is a paired cavity at the basis of the females' elytra; for isolating the spores present in the mycetangia, we removed the two elytra and placed their bases on malt agar (MA: 25 g malt extract, 20 g agar, 1 l deionized H_2O) petri-dish plates. Elytral mycetangia were too small to be dissected completely, so we cannot exclude that our isolations also contained fungi sticking to the upper and bottom sides of the elytra. Guts of the beetles were dissected and squashed in a sterile Petri dish. Gut material was then spread across the surface of MA plates using a sterile metal loop. All cultures were then incubated at 25°C in darkness for about two weeks, and purified by subculturing.

Fungal isolations from laboratory galleries

After introduction into tubes, the foundress usually bored an entrance tunnel and inoculated the medium with fungi. Females start egg-laying shortly after fungal layers appear on gallery walls (e.g. Kingsolver and Norris 1977). In *X. saxesenii* the foundress and/or her offspring will continue egg-laying as long as the ambrosia gardens proliferates (Biedermann, unpublished data). Previous laboratory studies have documented that on average four periods of gallery development can be discerned: (1) three to five days with only the foundress and eggs present, (2) at least ten days with foundress, eggs and immatures (larvae and pupae) present, (3) about 40 days with eggs, immatures and adult offspring present, and (4) the nest-leaving phase, when the foundress has died and offspring have matured and gradually disperse (Biedermann et al. 2011). We timed our sampling of brood and fungi in accordance with this gallery development pattern: we dissected eight galleries in period 1, ten galleries in period 2, and nine galleries in period 4. We performed no dissections during period 3, because our sample size was limited and we expected only minor changes in the abundance of fungi between periods 2 and 3 relative to the periods before and after (as only the number of adults changes between period 2 and 3). Additionally, we dissected eight galleries that did not produce brood within one month post-introduction of the female, and nine galleries where all larvae died. From each of the galleries, we took eight samples from the gallery wall of the entrance tunnel (which is the oldest part of the gallery) and eight samples from the gallery wall of the brood chamber (where most inhabitants were present), using a sterile needle. These samples were used to determine the prevalence of the respective fungi within the sampled galleries (see "Statistical analysis" below). Four of these samples we placed on MA and four on cycloheximide-streptomycin malt agar (CSMA: 10 g malt extract, 15 g agar, 20 ml filter sterilized CSMA stock solution containing 2 mg of Cycloheximide and 1 mg Streptomycin, 1 L deionized H_2O) plates. MA is an unselective medium

for growing fungi, whereas CSMA selectively supresses most fungi except species that are cycloheximide tolerant like ophiostomatoid ambrosia fungi (= *Raffaelea* sp.; Cassar and Blackwell 1996).

Additional isolations

Single, live and healthy larvae from five different galleries were squashed in sterile Petri dishes and then plated on MA and CSMA agar using sterile metal loops. The body surface of living adult ambrosia beetles, especially of solitary foundresses, is frequently covered with a biofilm that can be harmful and kill beetles, if not groomed off by other group members (Biedermann and Taborsky 2011). We aimed to isolate the fungi forming this biofilm by scrapping parts off with a sterile needle from four living adult females where it was clearly visible (see Fig. 1) and which we had removed from their laboratory galleries. Four samples from each insect were plated on MA. The fungal composition of eight gallery dumps sampled during period 2 (i.e. frass and sawdust shuffled out of the nest by female beetles onto the surface of the medium) was analysed by plating four samples each on MA and CSMA.

Identification of filamentous fungus isolates

Fungi isolated from beetles were initially identified based on colony characteristics in culture (i.e. morphology and color of mycelium and fruiting structures). Representative samples from single spore isolates were used for DNA sequencing. To extract DNA, a small amount of mycelium and conidia was scraped from the surface of young, relatively unmelanized colonies growing on MA, or hyphae were taken from cultures grown in 2% malt extract broth (MEB). The mycelium was macerated in 200 µl PrepMan Ultra (Applied Biosystems, USA), incubated at 95°C for 10 min, and then centrifuged. The supernatant containing DNA was then used for PCR amplification of a portion of the ribosomal RNA encoding region and partial b-tubulin gene with the primer pairs ITS3 (White et al. 1990) and LR3 (Vilgalys and Hester 1990), and Bt2b (Glass and Donaldson 1995) and T10 (O'Donnell and Cigelnik 1997). PCR conditions used have been described previously (Six et al. 2009). Amplicons were purified using a High Pure PCR Product Purification Kit (Roche, Germany) and sequencing was performed on an ABI 3130 automated sequencer (Perkin–Elmer Inc, USA) at the Murdock Sequencing Facility (University of Montana, Missoula, MT USA). DNA sequences of representative isolates were deposited in GenBank (Table 1). Contigs of forward and reverse

sequences obtained with each primer pair were aligned in MEGA5 (Tamura et al. 2007). BLAST searches were done with sequences of each isolate in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). Cultures of the three most consistent species from this study were deposited in the culture collection of Diana Six (DLS) at the University of Montana, Missoula, MT, USA, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). The remaining isolates were deposited in the culture collection of Diana Six for further study.

Statistical analysis

Using all 16 samples taken from each laboratory gallery we determined whether a fungus was present or not in the eight samples taken from the entrance tunnel and the brood chamber of each gallery (i.e., a binomial variable). Additionally, we estimated the prevalence of each fungal species per gallery (i.e., the rate of detection between 1/16 and 16/16). For each fungal species we analysed how its presence and prevalence (dependent variables) were affected by the culture medium (MA, CSMA), the location within the gallery (brood chamber, entrance tunnel) and the period of offspring development (foundress with eggs, foundress with larvae, only adult progeny; fixed factors) by controlling for gallery of origin (random factor). Using data from the first two periods only (foundress with eggs, foundress with larvae) we also tested if numbers of eggs and larvae correlated with the abundance of fungi. Analyses were done using generalized linear mixed models (GLMMs; lmer in R) or generalized linear models (GLMs; glm in R) using R (Version 2.12.1; R Development Core Team 2008). GLMMs are an extension of GLMs and allow for controlling the variation between observations from a single gallery. In most cases this was necessary because of variation in sample sizes between galleries, as plates had to be excluded from the analyses if they did not yield microbial growth.

Results

Our morphological data in combination with DNA sequencing revealed six species of filamentous fungi to be associated with *X. saxesenii* (Table 1). As expected, *R. sulfurea* was regularly isolated from *X. saxesenii* bodies and galleries. Both sequences (ITS and β-tubulin) generated for this fungus matched those deposited in GenBank (accessions of subject sequences) for this species at 100% (Table 1). The isolates also exhibited the distinct morphology of this species including deeply melanised hyphae forming sporodochia and colonies tinged with a deep sulphur yellow colour. ITS sequences for the other fungus isolated regularly most closely matched sequences for *Fusicolla acetilerea* (formerly *Fusarium merismoides* var. *acetilereum*) deposited in GenBank (100%) (Table 1). There was no match for the β-tubulin sequence generated for this fungus (closest match, 85% to

Fusarium domesticum) (Table 1). The dark morphospecies isolated from gallery dumps and biofilms found on dead insects matched morphological descriptions for *Paecilomyces variotii*. Sequences for this fungus also matched those for this species in GenBank (ITS, 100%; β-tubulin, 99% p difference) (Table 1). Less commonly isolated fungi included *Penicillium decaturense* (ITS and β-tubulin 100% match in GenBank,), Unknown sp. A, and Unknown sp. B. For Unknown sp. A, we were unable to amplify more than 125 bp of the ITS region. There was no informative match (>90%) to the β-tubulin sequence for this fungus in Genbank. For Unknown sp. B there was no informative match in GenBank to either the ITS or β-tubulin sequences.

Overall, *R. sulfurea* (generalized linear mixed models (GLMM): $p < 0.001$), Unknown sp. A (only on CSMA) and Unknown sp. B ($p = 0.003$) were more commonly detected on CSMA than on MA, whereas the opposite was true for *F. acetilerea* ($p < 0.001$; Table S1). The other species were isolated equally often from CSMA and MA.

Mycetangially-transmitted fungi

F. acetilerea dominated in mycetangia of all thirteen dissected females and was found in six of their guts. *R. sulfurea* was present in mycetangia of only one of these females, but was isolated from 9 of 13 female guts (Fig. 2).

Fungus dynamics in relation to development of progeny

R. sulfurea and the *F. acetilere*a dominated the gardens of freshly founded galleries after the foundresses had started to lay eggs (period 1, Fig. 2). Egg numbers tended to increase with increasing prevalence of *R. sulfurea* (GLMM: $p_{prevalence} = 0.09$; Table 2). The presence and prevalence of single fungal species in unsuccessful galleries without any eggs did not differ from galleries with eggs ($p = 0.26 – 1$, depending on species).

All six species of fungi were isolated from samples taken during the period after eggs had hatched (period 2). *F. acetilerea* increased in its presence (period 1 vs. 2: $p_{presence} = 0.02$; Table S1). However, this did not relate to larval numbers ($p = 0.92$; Table 2). Instead, larval numbers were positively correlated with the prevalence of *R. sulfurea* ($p = 0.035$), and tended to correlate negatively with the prevalence of *P. variotii* ($p = 0.063$; Fig.3, Table 2). The latter trend disappears, however, if one outlier ($x = 0$, $y = 31.25\%$) is removed from the data (Fig. 3). Fungus composition (presence and prevalence of single species) of galleries in which all larvae died during development did not differ from galleries with successfully developing larvae ($p = 0.17 – 1$, depending on species; details not shown).

Abundance of *R. sulfurea* (period 1 vs. 4: $p_{prevalence}$ = 0.028) and *P. variotii* (period 2 vs. 4; $p_{prevalence}$ = 0.001) were significantly lower after maturation of all offspring (Table S1). Only presence of *Pe. decaturense* increased towards this period (period 1+2 vs. 4: $p_{presence}$ = 0.02). Data for *F. acetilerea* were somewhat contradictory; the number of galleries where it was present decreased (period 2 vs. 4: $p_{presence}$ = 0.03), whereas its prevalence within galleries increased (period 2 vs. 4: $p_{prevalence}$ = 0.002; Table S1).

Fungal composition in relation to location

R. sulfurea ($p_{prevalence}$ < 0.001, $p_{presence}$ = 0.12) and *F. acetilerea* ($p_{prevalence}$ = 0.002, $p_{presence}$ = 0.08) were more common in the brood chamber than in the entrance tunnel of the galleries, while the opposite result was determined for *P. variotii* ($p_{prevalence}$ < 0.001, $p_{presence}$ = 0.01; Table S1). *P. variotii* was the dominant species growing in the dumps of the beetles (present in 7 of 8 galleries), followed by *F. acetilerea* (in 2 of 8 galleries), Unknown sp. B (in 1 of 8 galleries) and *Pe. decaturense* (in 1 of 8 galleries; Fig. 2).

Fungi on the body of adults

Lonesome foundresses were regularly found to be overgrown with a thin layer of fungi. If they were not able to successfully produce offspring (who would have groomed off this layer), this likely led to the death of these females, because this layer becomes so thick that it constricts movements of beetles through the tunnels (Biedermann and Taborsky 2011). *P. variotii* (present on 4 of 4 of these beetles) was the main component forming this layer, but *F. acetilerea* (in 2 of 4 beetles; Fig. 2) was also found.

Discussion

The mutualistic associates of *X. saxesenii*

R. sulfurea and *F. acetilerea* were the only fungi isolated from the spore-carrying organs of dispersing females ready to found new fungus gardens (Fig. 2, Table 3). Their elytral pouches (mycetangia) contained mostly spores of *F. acetilerea*, whereas *R. sulfurea* dominated in gut samples, which led to the initial high prevalence of these two species in the fungal gardens and during the period of larval development. The primary food fungus, *R. sulfurea,* formed thin ambrosia layers that were fed upon by the adults (Fig. 4; Biedermann and Taborsky 2011). While fungus farming ants and termites actively pick symbiont propagules to found new fungus gardens (Mueller et al. 2005), ambrosia beetles passively take up spores into external mycetangia from the surrounding environment when

moving within their natal nest before dispersal (Beaver 1989). At that time (which is the period when only adult progeny is present), galleries were already heavily infested by four other non-mutualistic fungi, Unknown sp. A, Unknown sp. B, *Pe. decaturense* and *P. variotii*. The increased prevalence of *R. sulfurea* in the gut relative to mycetangia might indicate that this main mutualist is actively taken up earlier than at the time of dispersal. Selective substances produced in the gut lumen and by numerous glands lining the beetles` mycetangia (Schneider and Rudinsky 1969; Schneider 1991) on the other hand likely also assure the exclusive transmission of *R. sulfurea* and *F. acetilerea.* In summary, this confirms the presumed simultaneous existence of two spore carrying modes in *X. saxesenii* (Francke-Grosmann 1975), and it suggests important roles for these two fungi in the life-cycle of this beetle.

Several observations suggest a strong mutualistic role of *R. sulfurea* in this system. First, it prevailed in galleries shortly after their foundation and throughout brood development, where it formed characteristic ambrosial layers of densely packed conidia with large nutritional conidiospores on the gallery walls (Fig. 4). Ambrosia layers are predominately formed within the brood chambers, where adult daughters and larval offspring aggregate and crop off the nutritional conidia (Biedermann and Taborsky 2011). Second, the number of eggs produced by the foundress and the number of larvae tended to correlate positively with the abundance of *R. sulfurea.* Third, the abundance of *R. sulfurea* was lowest in galleries with only adult progeny, suggesting that egg production ceased when productivity of this fungus dropped below a certain threshold (Table 3). Fourth, *R. sulfurea* has been isolated from mycetangia and galleries of *X. saxesenii* originating from locations across the US and Europe (Francke-Grosmann 1956; 1975; Batra 1967; Roeper et al. 1980; Roeper and French 1981). *Raffaelea* (Ascomycota: Ophiostomatales) and *Ambrosiella* (Microascales) species are also the primary mutualists of many other temperate ambrosia beetles (Roeper and French 1981; Farrell et al. 2001; Harrington et al. 2010).

F. acetilera was part of the fungal biofilm that sometimes formed on adults and was also common within brood chambers during development of larvae. Related species (the genus *Fusarium sensu lato*) appear to be extremely common in ambrosia beetle gardens and play different roles for their hosts (e.g. Norris 1979). While *Fusarium solani* associated with *Dendroctonus frontalis* is weakly pathogenic to its host (Moore 1973), other strains of *F. solani* isolated from galleries of *Anisandrus dispar* and *Xyleborus ferrugineus* (Zimmermann 1973; Norris 1979) as well as *Fusarium merismoides* isolated from *Platypus quercivorus* (Platypodinae; Qi et al. 2011) apparently have nutritional functions for their hosts. Our observations suggest a secondary mutualistic role of *F. acetilerea* for *X. saxesenii*, but experimental studies are needed to determine whether this is actually the case.

The cultivation of two or more mutualists may be common in ambrosia beetles (Norris 1979; Haanstad and Norris 1985; Harrington and Fraedrich 2010; Endoh et al. 2011), against the predictions of hypotheses regarding the formation and maintenance of mutualisms. Symbiont competition can generate selection for symbiont traits that enhance their competitive ability at the cost of harming the host (Frank 1996; Mueller 2002). Additionally, there should be strong selection for a 'best symbiont', over time leading to its fixation with a host. However, while some symbioses, including fungus-farming ants and termites, involve only one main mutualistic partner (e.g. Mueller et al. 2005), many others involve multiple symbionts, indicating mechanisms that allow their coexistence (Six 2012). In the case of symbionts in ambrosia beetle gardens, niche differences of the various fungi may reduce competition. If the fungi exploit different resources in the tree, this may alleviate selection against any one partner and help to maintain a community of symbionts rather than a single fungal partner. In *X. saxesenii,* for instance, one fungus species might serve as food for the mycetophagous adults and the other one as food for the xylomycetophagous larvae (De Fine Licht and Biedermann 2012). Cooperation between symbionts is also possible. Laboratory studies showed that *R. sulfurea* requires exogenous B-vitamins to grow, which might be provided by other microbes (filamentous fungi, yeasts or bacteria; R.A. Roeper, personal communication). In *Dendroctonus* bark beetles (Scolytinae), the possession of several apparently redundant fungal symbionts with differing environmental tolerances may reduce the risk of the host being left aposymbiotic, when environmental conditions shift over a season and from year to year (Six and Bentz 2007). Experimental studies are needed to clarify the roles and interactions of the various symbionts associated with bark and ambrosia beetles.

Other fungal associates

Species of the anamorphic genera *Paecilomyces* and *Penicillium*, as well as two unknown species were also associated with *X. saxesenii,* without being transmitted by founder females in their spore-carrying organs (Fig. 2, Table 3). Instead, spores of such fungi have been found to be vectored in small quantities on females` body surfaces (Francke-Grosmann 1967). Unknown sp. A was isolated at low frequencies from all gallery-classes and also from larval bodies. The presence of this fungus did not affect adult beetles or larvae in this study. *Pe. decaturense* and *P. variotii* predominated in old galleries, at the entrance tunnel and in gallery dumps. *Pe. decaturense* has only been isolated previously from a wood decay fungus (Peterson et al. 2004) and is known to produce anti-insect compounds (Zhang et al. 2003). *Penicillium* species often compete with insects for ephemeral resources and thus regularly produce compounds against insect feeding (Peterson et al. 2004; Rohlfs and Churchill 2011). *Penicillium* species have been frequently reported from old galleries of *X.*

saxesenii and have been regarded as weak antagonists (Fischer 1954; Francke-Grosmann 1975). However, we found no negative effects of *Pe. decaturense* on the host beetle. *P. variotii* appears to act as a weak pathogen for *X. saxesenii*: Its abundance tended to negatively correlate with larval numbers and it formed a fungal biofilm on the surface of adult beetles that can be deadly if not groomed off by group members (Fig. 1). In a previous study we found this biofilm to have caused the death of at least 7 out of 29 females, likely not because mycelium enters the body, but rather because it constricts female movements, which leads to females getting stuck within the narrow tunnels (Biedermann and Taborsky 2011). The genus *Paecilomyces* includes many entomopathogenic species and also plant saprobes belonging to the earliest colonizers of recently dead plants (e.g. Kim et al. 2001; Tang et al. 2005).

Fungus dynamics in a laboratory setting vs. field galleries

Ambrosia beetles live in the wood of trees, where they can only be studied by destructive gallery dissection. Thus, a laboratory setting was required to study the fungus dynamics in relation to the dynamics of the beetles' life history within galleries. It is intrinsic to all laboratory studies, however, that results might be influenced by differences between laboratory and field conditions. Our artificial breeding medium, for example, is richer in nutrients and moisture than natural wood (Saunders and Knoke 1967). Therefore, it is important to consider whether these differences could have influenced the conclusions of our study. Supporting our laboratory results is the observation that total numbers of offspring of *X. saxesenii* in field and laboratory galleries are almost identical (Biedermann et al. 2009). Also, while different substrate conditions might influence the prevalence of particular fungi relative to others, they should not affect within species dynamics (e.g., the time course of prevalence in dependence of gallery stage and composition). Thus, we believe that our conclusions are generally valid but the results on relative prevalence of different species of fungi should be interpreted with caution.

Can beetles influence the community of their gardens?

Larvae and adult *X. saxesenii* constantly remove the growth of *F. acetilerea* and *P. variotii* from their body surface by grooming each other. They also constantly crop their gardens and hinder the spread of pathogens by dumping old sawdust, faeces, fungal material and dead individuals out of the gallery entrance (Biedermann and Taborsky 2011). If larvae and adults are removed from a gallery, its fungus gardens are overrun by saprobic fungi (normally coexisting at low levels) within 1-2 days (Leach et al. 1940; Batra 1979; Norris 1979; Biedermann and Taborsky 2011), which demonstrates that beetles play an active role in maintaining the composition of their gardens. As

mechanical removal is likely to be only partially effective against 'weed' fungi, beetle-associated antibiotics-producing bacteria may play a role in controlling weeds and pathogens, like in other fungus-culturing insects (Mueller et al. 2005). In *Dendroctonus* bark beetles, several bacterial groups have been found to reduce the growth of antagonistic fungi (Scott et al. 2008), and some of them are actively applied with oral secretions during specialised cleaning behaviours by the adults (Cardoza et al. 2006). *Streptomyces griseus,* which is known to produce antibiotics, has been recently isolated from *X. saxesenii* galleries (Grubbs et al. 2011). Whether and how bacteria influence the composition of ambrosia gardens remains to be investigated.

This is the first study reporting correlative evidence for fitness effects of a fungal consortium on an ambrosia beetle. A relatively high number of filamentous fungi are regularly associated with the ambrosia beetle *X. saxesenii*. Interestingly, most of the genera of secondary fungal flora found in this study have been isolated also from nests of fungus-growing ants (Rodrigues et al. 2008; 2011): *Fusarium sensu lato*, *Paecilomyces* and *Penicillium* have been frequently isolated from different attine ant species. These genera are also often associated with plants, either as endophytes or early saprobes. Thus, these fungi are likely present in the plant material the ants collect to provision their gardens (Rodrigues et al. 2011). In the case of ambrosia beetles, the fungi must be vectored by the dispersing females, or enter via the entrance hole after excavation, even if this possibility is unlikely. The absence of a strong association between the secondary associates and the farming insects in both systems suggests that most of these filamentous fungi are transient components of the gardens. This does not mean, however, that secondary microbes do not influence insect-fungus symbioses (Silva et al. 2006).

Our study revealed six fungal species within ambrosia beetle gardens (Table 3), which is at least ten times less than the number of species isolated from fungus-growing ant gardens (between 66 and 106 fungal species, depending on the ant species; Rodrigues et al. 2011) and also much less than the species numbers isolated from fungus-growing termite gardens in the field (Thomas 1987; Guedegbe et al. 2009). These apparent differences between beetles and ants / termites may, in part, reflect differences between laboratory and field settings; however, a more important reason may be that beetle galleries are a much more closed system than the ant and termite nests (U.G. Mueller, personal communication). Ants and termites build their nests in soil, which is heavily colonized by microbes. They also leave the nest regularly to forage and thus are exposed to many kinds of contaminants which they may bring back to their nest. Additionally, they use substrates that contain endophytic and epiphytic microbes to feed their gardens. Ambrosia beetles, in contrast, tunnel into dying or recently dead wood, a substrate that is much less contaminated by microbes than soil. They also do not leave and re-enter their nests or introduce material from outside the nest. Furthermore,

beetle galleries are relatively short-lived, which reduces the time for additional fungi to enter the system. Thus, it seems that ambrosia beetles have a greater ability to protect their gardens from foreign fungi than do the fungus-farming ants and termites. Because of this, sophisticated techniques for weeding and disinfection like those observed in ant gardens (e.g. Currie et al. 1999; Currie and Stuart 2001; Boomsma and Aanen 2009) might not be needed in beetle gardens. The biggest threats to ambrosia gardens are desiccation (cf. Fischer 1954) and probably diminishing nutrients, so recycling of excretions may be important. Indeed, there is some evidence of nutrient cycling between beetles and fungi (Kok and Norris 1972a; 1972c; Biedermann and Taborsky 2011).

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Table 1. DNA regions sequenced, GenBank accession numbers, and culture accession numbers for fungi isolated from *Xyleborinus saxesenii* in this study.

Fungus species	Gene region	GenBank accession no.	Closest match GenBank accession no. (% similarity)	CBS Collection	DLS Collection
Raffaelea sulfurea	ITS	JX565086	*Raffaelea sulphurea* EU984292 (100)	CBS 132735	DLSPB 146
	β-tubulin	JX565092	*R. sulphurea* EU977467 (100)		
*Fusicolla acetilerea**	ITS	JX565088	*Fusarium mesmeroides* var. *acetilereum* EU860058 (100)	CBS 133245	DLSPB 148
	β -tubulin	JX565095	*Fusarium domesticum* EU926353 (85)		
Paecilomyces variottii	ITS	JX565087	*P. variotii* JF922032 (100)	CBS 132734	DLSPB 158
	β -tubulin	JX565093	*P. variotii* GU968679 (99)		
Penicillium decaturense	ITS	JX565090	*P. decaturense* AY313619 (100)		DLSPB 157
	β -tubulin	JX565091	*P. decaturense* JN606683 (100)		
Unknown sp. A	ITS		n/a (less than 125 bp)		DLSPB 152
	β -tubulin	JX565094	no informative match		
Unknown sp. B	ITS	JX565089	no informative match		DLSPB 151
	β -tubulin	JX565096	no informative match		

* formerly *Fusarium merismoides* var. *acetilereum*

Table 2. The relationship between the most common fungal species and the number of *X. saxesenii* offspring. Separate GLMMs were performed with an exchangeable correlation structure of the response variable within a cluster (gallery identity) to examine the potential influence of the prevalence of fungal species on offspring numbers, controlling for the influence of medium (CSMA – cycloheximide-streptomycin-malt agar; MA – malt extract agar). The potential effects on egg and larval numbers during particular periods of gallery development are shown (for graphical illustration see Fig. 3). Model coefficients are reported as coeff. ± se (standard error of the estimate), with the group in brackets in the first row of the model as the reference category (coefficient set to zero). A positive coefficient denotes a positive relationship; a negative coefficient denotes a negative relationship. Significant relationships ($p \leq 0.05$) are set in bold type and significant trends ($p \leq 0.01$) are underlined.

Fungal species	Parameters	coeff. ± se	t	p
Foundress with eggs, brood chamber				
Raffaelea sulfurea	Intercept of **prevalence** (CSMA)	-0.14 ± 1.45	-0.01	0.92
	Contrast CSMA vs. MA	-3.49 ± 1.04	-3.37	**<0.001**
	Number of eggs	0.9 ± 0.53	1.72	0.09
Fusicolla acetilerea	Intercept of **prevalence** (CSMA)	-22.9 ± 999	0	1
	Contrast CSMA vs. MA	Only present on MA		
	Number of eggs	-2.91 ± 4.24	-0.69	0.49
Paecilomyces variotii	Not present			
Foundress with larvae, brood chamber				
Raffaelea sulfurea	Intercept of **prevalence** (CSMA)	-0.15 ± 0.51	-0.3	0.77
	Contrast CSMA vs. MA	-2.49 ± 0.61	-4.08	**<0.001**
	Number of larvae	0.05 ± 0.02	2.11	**0.035**
Fusicolla acetilerea	Intercept of **prevalence** (CSMA)	-7.88 ± 4.82	-1.63	0.1
	Contrast CSMA vs. MA	13.6 ± 5.04	2.69	**0.007**
	Number of larvae	0.02 ± 0.21	0.1	0.92
Paecilomyces variotii	Intercept of **prevalence** (CSMA)	-0.86 ± 0.49	-1.76	0.08
	Contrast CSMA vs. MA	-0.46 ± 0.65	-0.71	0.48
	Number of larvae	-0.06 ± 0.03	-1.86	0.063

Table 3. Summary of observations of six fungal species isolated from galleries of *Xyleborinus saxesenii* reared in the laboratory.

Order	Species	Medium	Location	Fungal species dynamics during colony stages	Correlation with progeny numbers	Consistency (> 50% of samples)	Proposed association
Ophiostomatales	*Raffaelea sulfurea*	CSMA > MA	BC > MT[1]	foundress/eggs > adult progeny [1]	positive (larvae)	in gut, during foundress/eggs	mutualist
Hypocreales	*Fusicolla acetilerea*	CSMA < MA	BC > MT[1]	foundress/eggs < foundress/larvae [2] foundress/larvae < adult progeny [1] foundress/larvae > adult progeny [2]	Ns	in mycetangium	mutualist
	Penicillium decaturense	ns	ns	foundress/eggs < adult progeny [2] foundress/larvae < adult progeny [2]	Ns	-	commensal
Eurotiales	*Paecilomyces variotii*	ns	BC < MT[2]	not present during foundress/eggs foundress/larvae > adult progeny [1,2]	Ns	on adult bodies, in gallery dump	parasite
Unknown	Unknown	only	ns	ns	Ns	-	commens

	sp. A	on CSMA					al
	Unknown sp. B	CSMA > MA	ns	not present during foundress/eggs	Ns	-	commensal

Significant differences ($p < 0.05$) are given; ns indicates non-significance.

Abbreviations: CSMA – cycloheximide-streptomycin-malt agar, MA – malt agar; BC – brood chamber, MT – entrance tunnel; "…>…" /"… <…" denote the direction of the significant difference in the prevalence between the two media/locations/gallery classes.

[1] Significant difference in the prevalence of the fungus ($p \leq 0.05$).

[2] Significant difference in the presence of the fungus ($p \leq 0.05$).