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A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts

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Thousands of species of ambrosia beetles excavate tunnels in wood to farm fungi. They maintain associations with particular lineages of fungi, but the phylogenetic extent and mechanisms of fidelity are unknown. We test the hypothesis that selectivity of their mycangium enforces fidelity at coarse phylogenetic scales, while permitting promiscuity among closely related fungal mutualists. We confirm a single evolutionary origin of the *Xylosandrus* complex—a group of several xyleborine genera that farm fungi in the genus *Ambrosiella*. Multi-level co-phylogenetic analysis revealed frequent symbiont switching within major *Ambrosiella* clades, but not between clades. The loss of the mycangium in *Diuncus*, a genus of evolutionary cheaters, was commensurate with the loss of fidelity to fungal clades, supporting the hypothesis that the mycangium reinforces fidelity. Finally, *in vivo* experiments tracked symbiotic compatibility throughout the symbiotic life cycle of *Xylosandrus compactus* and demonstrated that closely related *Ambrosiella* symbionts are interchangeable, but the probability of fungal uptake in the mycangium was significantly lower in more phylogenetically distant species of symbionts. Symbiont loads in experimental subjects were similar to wild-caught beetles. We conclude that partner choice in ambrosia beetles is achieved in the mycangium, and co-phylogenetic inferences can be used to predict the likelihood of specific symbiont switches.

1. Introduction

Partner fidelity and partner choice are prevailing models for explaining the evolutionary stability of mutualisms. Partner fidelity describes associations where individuals or genetic lines of mutualists repeatedly interact and enforce mutually positive outcomes through punishment, reciprocation and positive fitness feedbacks. Partner choice applies to associations where mutualists might not interact repeatedly, but positive outcomes are favoured when one partner can select good cooperators from multiple potential partners [1,2]. Although typically presented as alternative scenarios, the two models are not necessarily mutually exclusive. Partner choice as a means of partner specificity may actually drive fidelity when transmission is often, but not always vertical [3]. Specificity of partner choice is rarely perfect and the implications of variable specificity for long-term fidelity and coevolutionary processes are largely unknown.

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Specificity in mutualisms is a continuum, and many systems are not as specific as they may seem at first look [4]. Previous inferences of strict specificity have been overturned in some of the best studied mutualistic systems as a result of improved molecular-based taxonomy and co-phylogenetic studies that reveal host and symbiont switches over evolutionary time [5–9]. Similarly, strict specificity has also been refuted as a result of species invasion and subsequent new encounters between host and symbiont species [10–12]. These departures from strict specificity may be common because they are advantageous. While a degree of specificity may stabilize mutualisms by enforcing partner fidelity [1–3], strict specificity can be an evolutionary dead end. When species are absolutely dependent on other species, the extinction of one is inevitably followed by the extinction of the other [13]. Strict specificity also diminishes species' abilities to adapt to changing conditions, or oppositely, non-specificity allows populations and individuals to form associations that are best suited for current local conditions and maintain a broad niche breadth [14–17]. The degree of specificity in symbioses need to be considered in a phylogenetic framework because symbionts are more likely to switch among closely related hosts than distantly related hosts, and vice versa [18,19]. We hypothesize that this phylogenetic bias in specificity maintains coarse-scale phylogenetic congruence between hosts and symbionts in systems where long-term fidelity is enforced by the phylogenetic specificity of partner choice. Testing this prediction requires combined experimental and phylogenetic studies to characterize the degree of fidelity at ecological and evolutionary time scales, and identify their underlying mechanisms.

Few symbiotic systems are as amenable to comparative evolutionary studies as the ambrosia beetle–fungus system. Ambrosia beetles engage in the most diverse and widespread fungus-farming mutualism known. They comprise approximately 3400 species within two subfamilies of weevils (Curculionidae; Scolytinae and Platypodinae) [20]. Several clades of ambrosia beetles have independently evolved specialized pouch- or pit-like organs termed mycangia (singular: mycangium) which are used to transport actively growing and reproducing fungal propagules to newly established galleries [21–23]. In return for dispersal, the fungi produce specialized enlarged nutritious spores that comprise the majority or entirety of the beetle diet [21–23]. While some ambrosia beetles carry multiple related species within their mycangium [24,25], more commonly ambrosia beetles carry a single primary symbiont in their mycangium [26–28]. The phylogenetic extent of fidelity in ambrosia beetles and fungi and the underlying mechanism(s) are unknown. Likewise, the mechanisms that control fidelity are also poorly understood in other insect–fungus symbioses such as bark beetles [29], ants [5,30] and wood wasps [11,31].

In this study, we address multiple mechanisms that have been hypothesized to support fidelity in ambrosia symbioses using a newly developed experimental ambrosial study system, the *Xylosandrus* complex (Coleoptera, Curculionidae, Scolytinae) and *Ambrosiella* fungi (Fungi, Microascales, Ceratocystidaceae). The *Xylosandrus* complex is one of the most successful, widespread, well-studied and economically relevant lineages of ambrosia beetles, consisting of 111 described species in six genera in the tribe Xyleborini (*Anisandrus*, *Cnestus*, *Diuncus*, *Eccoptopterus*, *Hadrodemius* and *Xylosandrus*) that live in tropical to temperate forests around the world. While some studies have suggested that the *Xylosandrus* complex is polyphyletic [32–34], others have found monophyly albeit with limited taxon sampling [35,36]. The *Xylosandrus* complex also includes several species of widely introduced forest health and agricultural pests [37]. Females in this group possess large mesothoracic glandular mycangia just beneath the scutellum [21]; (figure 1). *Xylosandrus* complex species have each been found consistently associated with a single fungus species in the genus *Ambrosiella* (Microascales: Ceratocystidaceae) across multiple continents, even in areas where multiple *Xylosandrus* complex species co-infest the same trees [23,26,39–42]. It has been hypothesized that the mycangium is selective in the sense that it is an environment that is only habitable or accessible to particular lineages of symbiotic fungi [26], though there have been no previous experimental tests of the selectivity of the mycangium.

One genus in the *Xylosandrus* complex, *Diuncus*, lacks a mycangium [35]. *Diuncus* exemplify an evolutionary cheater strategy because they exploit the nutritious spores produced by ambrosia fungi, but do not reciprocate as fungal vectors. Instead, they bore their gallery near the gallery of other ambrosia beetles, and feed on the other beetle's fungus as the hyphae extend into their gallery [35]. The existence of fungal cheaters, fungi that infiltrate the mycangium of ambrosia beetles but do not produce nutritious spores in the gallery, has been suggested but not yet demonstrated [28].

We constructed the most extensive to date phylogenies of the *Xylosandrus* complex and *Ambrosiella* fungi, and statistically tested for the co-phylogenetic structure at multiple phylogenetic scales. Informed by observed co-phylogenetic patterns, we conducted the first symbiont switching experiments using laboratory raised aposymbiotic *Xylosandrus* ambrosia beetles to test the hypothesis that the mycangium provides a mechanism for partner choice and an intermediate degree of fidelity that is contingent on phylogenetic distances among potential symbionts. Our results reveal a driving mechanism behind the co-phylogenetic structure and patterns in ecological associations in the world's widest ranging and diverse fungus-farming mutualism and illustrate the utility of co-phylogenetic analysis for predicting lateral transmission of fungal symbionts among vector species.

2. Material and methods

(a) Cophylogenetic analysis

Sequences for five marker genes for beetles were obtained from specimens and databases: mitochondrial gene cytochrome oxidase I (COI), nuclear large subunit ribosomal gene (28S), elongation factor 1-alpha (EF1α), a gene which encodes carbamoylphosphate synthetase, aspartate transcarbamylase and dihydroorotase (CAD) and arginine kinase (ArgK). For *Ambrosiella* fungi, we targeted three marker genes: the nuclear large subunit ribosomal gene (28S/LSU), trans-elongation factor 1-alpha (TEF1α) and RNA polymerase II subunit 1 (RPB1). Because the monophyly of *Xylosandrus* and similar genera has not been consistently found in previous taxon-rich phylogenetic studies of Xyleborini [32,33] we included 17 other Xyleborini genera to test for monophyly of the *Xylosandrus* complex. We also included *Coccotrypes* (Dryocoetini), which is probably the ancestral group of all Xyleborini, and *Dryocoetiops* (Dryocoetini) to root our beetle phylogeny. Monophyly of *Ambrosiella* associated with *Xylosandrus* has been previously demonstrated [26].

In addition to all available sequences on GenBank, we obtained 19 beetle sequences and 12 fungus sequences from

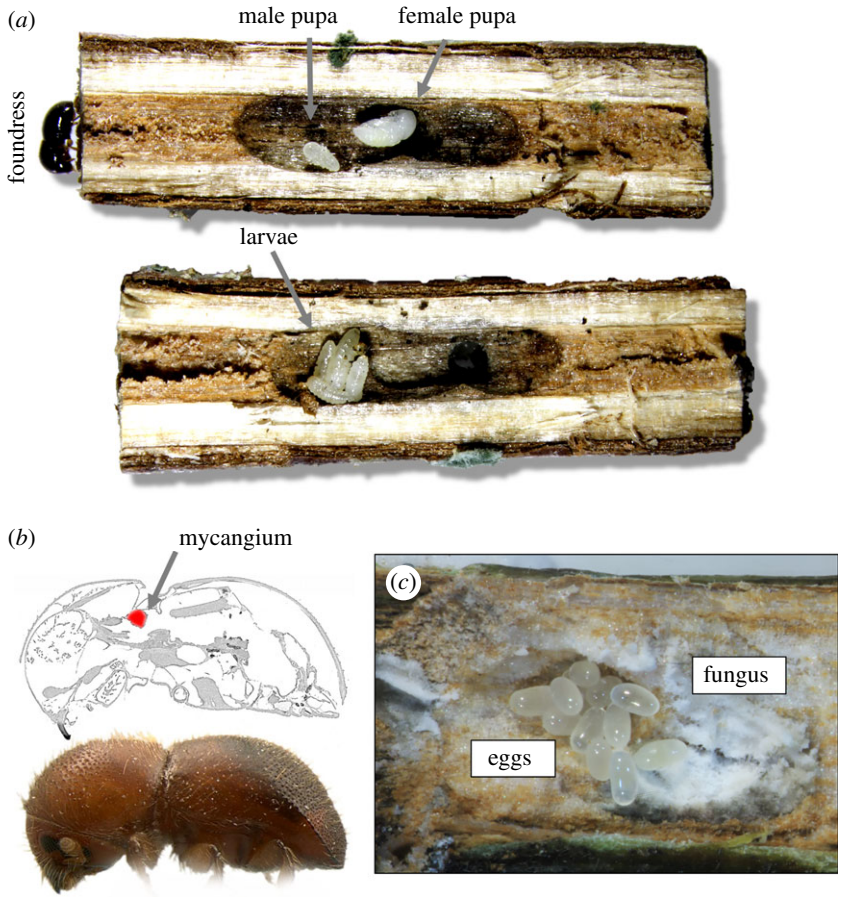

Figure 1. Typical *Xylosandrus* ambrosia beetle life history. (*a*) A foundress excavates a gallery in the pith or xylem of a twig or branch. She produces daughters and one to several haploid sons. Males are small and flightless, and mate with their sisters and mothers [38]. Mated diploid females disperse and establish new galleries. Just prior to dispersal the females' mycangia (*b*) rapidly fills with budding fungal spores, which are used to propagate a fungal garden in newly established galleries. Shown is a micro CT scan cross section of *Xylosandrus crassiusculus* with the mycangium highlighted in red. (*c*) Dispersed females initiate new galleries and oviposit after the establishment of ambrosia fungi, visible as a dense mat of enlarged nutritious spores surrounding a clutch of *Xylosandrus compactus* eggs in a natural gallery in red bay *Persea borbonia*. (Online version in colour.)

specimens of seven beetle species stored in the University of Florida Forest Entomology cryo-preserved collection (see electronic supplementary material, tables S1 and S2). See electronic supplementary material for DNA extraction methods. GenBank accession numbers are listed in the electronic supplementary material, tables S1 and S2.

Phylogenies for ambrosia beetles and ambrosia fungi were inferred with the same methods (see electronic supplementary material). The beetle phylogeny was pruned to only include beetles for which observations of fungal associations were available, and reciprocally the fungus tree was pruned to only fungi associated with represented beetles. Associations for beetles in the genus *Diuncus* were inferred from published and unpublished observations of gallery parasitism on other species in the *Xylosandrus* complex with known fungal associates [35]. Even though they lack mycangia, *Diuncus* were included in the graphical representation of cophylogenetic relationships to see if the species that they parasitized were restricted to those that farm a particular lineage of fungi, or conversely if fidelity to fungal lineages was lost with the mycangium.

Phylogenetic congruence between the *Xylosandrus* complex and *Ambrosiella* fungi was visually assessed by constructing a co-phylogenetic tanglegram using the cophylo() function of the ape package [43] for R [44], and rotating nodes to find the paired topologies with fewest intersecting ties. Pruned trees were converted to pairwise patristic branch length distance matrices for co-phylogenetic analysis using the cophenetic() function in ape [43]. We tested for significant congruence using a null model test of cophylogenetic congruence based on Procrustean analysis (paco [45]), implemented using the PACo() function, of the paco package [46] for R. After preliminary analysis indicated that all or most of the phylogenetic congruence between beetles and fungi corresponded to the deepest division in the fungal tree, we conducted subsequent paco analyses for all links within each of two major fungal clades (hereafter the *beaveri* clade and the *xylebori* clade) separately to determine if there was congruence at finer phylogenetic scales within the major clades of *Ambrosiella*. For all paco analyses, we applied a symmetrical Procrustean analysis and used the quasi-swap matrix permutation algorithm [47], which maintains row and column sums, to create 1000 permutations of the interaction matrix, and provides a conservative test of the null hypothesis of no congruence, and does not require prior assumptions about which lineage has tracked the other over their coevolutionary history [46,48].

(b) Symbiont switching experiments

(i) Beetle collection and fungal isolation

During the spring of 2016, we obtained isolates of *Ambrosiella* fungi from the mycangia of *X. compactus*, *X. amputatus* and *X. crassiusculus* in Gainesville, Florida, USA. We also obtained isolates from the mycangia of *X. discolor* in Tam Dao National Park, Vinh Phuc Province, Vietnam and from *X. germanus* collected in Madison, Wisconsin, USA. Dispersing *Xylosandrus* beetles were collected alive using bottle traps [49] and Lindgren funnel traps baited with 95% ethanol. Fungal isolation, estimation of spore abundance, purification, DNA extraction, PCR and DNA

barcode identification followed the methods of Bateman *et al*. [50]. To extract fungi from the mycangium, beetles were impaled through the head and pronotum with insect mounting pins. Gentle pressure was applied to the scutellum to evert the prothoracic mycangium, expelling the paste-like fungal spore mass when present. Mycangia were confirmed to be empty when fully everted without expelling a visible spore mass (see electronic supplementary material, video supplement V2). We considered a mycangium with any visible spore mass to be 'full', recognizing that full mycangia will vary in the number of propagules they contain. Spore masses were aseptically transferred to a 1.5 ml tube with 500 µl sterile phosphate buffered saline, vortexed for 30 s, and plated on standard potato dextrose agar at 0.1, 0.01 and 0.001 spore dilutions. Plates were incubated for one week at 25°C prior to colony morphotype assignment, estimation of colony forming units (CFU) and subculture. Subcultures were incubated one week further, inspected for purity and used for extraction of genomic DNA. From genomic DNA, we amplified and sequenced translation elongation factor one alpha (TEF1α) using the primers EFCF1.5 and EFCF6 [51] for identification by comparison to sequences of type materials available on GenBank (electronic supplementary material, table S1). We recovered isolates of *Ambrosiella xylebori*, *A. nakashimae* and *A. roeperi* from *Xylosandrus compactus*, *X. amputatus* and *X. crassiusculus*, respectively. We isolated *A. beaveri* from *X. discolor* and *A. grosmanniae* from *X. germanus*. All of these associations match previous accounts [26,39,40,42,50].

(ii) Beetle development and mycangium assays

Beetles were reared from eggs and first instar larvae in artificial galleries experimentally inoculated with one of three *Ambrosiella* species. See electronic supplementary material for artificial gallery construction and inoculation methods. We tested the typical symbiont *A. xylebori*, the Vietnamese isolate from *X. discolor* (*A. beaveri*) and *A. grossmaniae* from *X. germanus* collected in Wisconsin, USA. Eggs and larvae were harvested from wild active galleries collected in Gainesville Florida, USA ($n = 74$). Eggs and first instar larvae were placed on sterile moistened filter paper, washed by dripping sterile PBS via micropipette and incubated for 24 h before being washed again. We placed three to five randomly selected eggs and first instar larvae (in combination) in each colonized experimental gallery, then incubated at 25–30°C and 90–100% RH, with a 12 h d^{-1} indirect full spectrum LED light and monitored daily for emergent adults.

In the mycangium assay, we reared adult beetles from pupae in experimental galleries to determine if the mycangium of *X. compactus* could support the growth and transportation of alternative ambrosial species. Beetles were reared on the typical symbiont (*A. xylebori*; $n = 14$), one of four alternative symbionts *A. roeperi* ($n = 10$), *A. grosmanniae* ($n = 12$), *A. beaveri* ($n = 7$), *A. nakashimae* ($n = 11$), or in negative control galleries without any inoculated fungi ($n = 5$). Pupae were harvested from laboratory reared broods (see electronic supplementary material). We placed two to three female pupae in each experimental gallery and monitored daily for emergent adults. The mycangia of emergent adults were immediately examined for a fungal spore mass as described above. When a spore mass was present, spore abundance and identity were determined by dilution plating and DNA barcode identification as described above. Between 4 and 12 isolates were sequenced from every emergent adult beetle with a full mycangium to determine if mycangial contents were uncontaminated monocultures. After eight weeks, galleries were opened to confirm the mortality of remaining beetles. To determine if spore loads in our experiment were similar to naturally reared beetles, we used the same methods to identify and quantify spore loads in six wild-collected dispersing black twig borers.

For the beetle development assay, we tested for an effect of fungus species on the probability of survival and development to adult using a binomial generalized linear model (GLM). We used the typical symbiont (*A. xylebori*) as the reference group and the number of adults recovered versus the number of mortalities per gallery as our response variable. We also used binomial GLM to test for differences in the proportions of emerging adults that had full mycangia among fungal species treatments in the development and mycangium assays. For these analyses, our response variable was the number of emergent adults with full versus empty mycangia, per gallery. We conducted binomial GLM at the fungus species level to test for individual differences between the typical symbiont, *A. xylebori* (reference group), and each other *Ambrosiella* species. We also conducted a clade-level generalized linear mixed model (GLMM), in which all alternative symbionts from the same clade of *Ambrosiella* as the typical symbiont were compared to *Ambrosiella* from the alternative clade, with a random intercept for fungal species. We also tested for differences in symbiont survival and abundance by comparing colony forming unit (CFU) counts estimated from full mycangia between all experimental treatments, and beetles captured wild in flight. For this, we used a negative binomial GLM implemented by the glm.nb() function of the MASS package [52] with field collected beetles as the reference group. A negative binomial model was chosen because of high overdispersion in the CFU data. GLM analyses were implemented using the glm() function in the R stats package [44] and GLMM using the glmer() function of the lme4 package [53].

3. Results

(a) Phylogenetic analysis

Phylogenetic analysis found monophyly of the *Xylosandrus* complex containing *Anisandrus*, *Cnestus*, *Diuncus*, *Eccoptopterus*, *Hadrodemius* and *Xylosandrus* (electronic supplementary material, figure S1). All genera were found to be monophyletic except *Anisandrus*. The mycocleptic genus *Diuncus* was nested within mycangia-possessing species, indicating that the mycangium and associated tuft of setae on the pronotum were secondarily lost in this lineage of evolutionary cheaters. The phylogeny of *Ambrosiella* revealed two major clades, the *xylebori* clade and the *beaveri* clade. The *xylebori* clade contained *A. catenulata*, *A. cleistominuta*, *A. batrae*, *A. grossmaniae*, *A. hartigii*, *A. roeperi* and *A. xylebori*, as well as two undescribed *Ambrosiella* species sequenced directly from the mycangia of *X. morigerus* and *E. gracilipes*. The *beaveri* clade contained several genetically similar fungi including *A. nakashimae* and multiple genotypes identified as *A. beaveri* in previous work [26,39], which may represent multiple closely related species.

(b) Co-phylogenetic analysis

Co-phylogenetic congruence between the ambrosia beetle and ambrosia fungus phylogenies was highly significant when analysed across all clades (procrustean test of phylogenetic congruence; $p < 0.001$; figures 2 and 3*a*). Most of the congruence was derived from beetle genera that had exclusive associations to members of either of the two major fungal clades; all members of two clades of beetles were associated with fungi in the *beaveri* clade, one comprises *Hadrodemius* and *Cnestus*, the other comprises four species of *Xylosandrus*: *X. amputatus*, *X. brevis*, *X. discolor*, and *X. mancus*. All other beetles, excluding the mycocleptic parasites in *Diuncus*, were exclusively associated with *xylebori* clade fungi. In contrast to

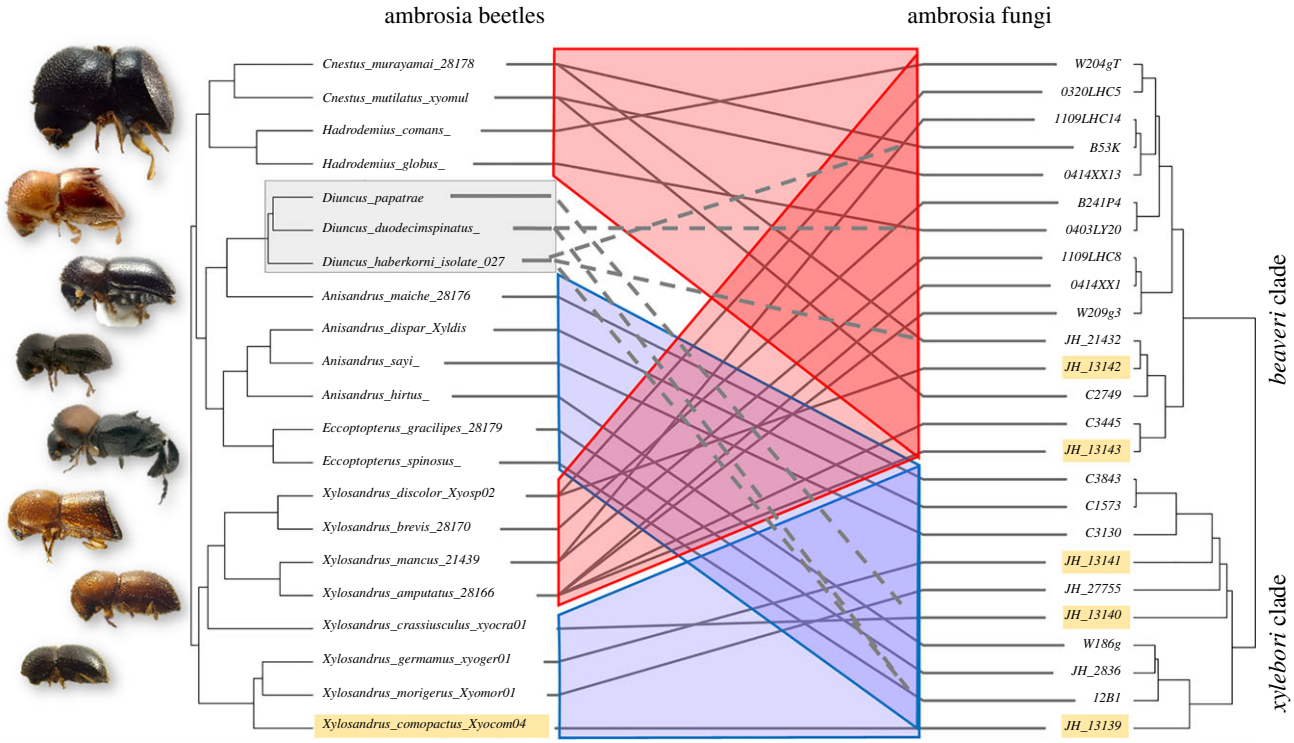

Figure 2. Coarse phylogenetic congruence in beetles of the *Xylosandrus* complex and *Ambrosiella* fungi. Tanglegram shows phylogenetic congruence among ambrosia beetles and ambrosia fungi; two clades of beetles are exclusively associated with the *beaveri* clade of *Ambrosiella* fungi (highlighted in red). The remainder is associated with the *xylebori* clade (blue), except for the fungus-stealing cheaters in the genus *Diuncus*. Dotted lines indicate inferred relationships with *Ambrosiella* farmed by parasitized beetle species. Unlike all other *Xylosandrus* complex beetles with mycangia, *Diuncus* was associated with both major *Ambrosiella* clades. Names of fungal isolates and beetle used in symbiont switching experiments are highlighted; JH_13142 = *A. beaveri*, JH_13143 = *A. nakashimae*, JH_13141 = *A. grosmanniae*, JH_13140 = *A. roeperi*, JH_13139 = *A. xylebori*. (Online version in colour.)

the strong coarse-scale co-phylogenetic pattern, we found no significant congruence at finer phylogenetic scales within *xylebori* clade associations ($p = 0.116$), nor within *beaveri* clade associations ($p = 0.559$).

(c) Development assay

Adult *X. compactus* beetles were successfully reared from eggs and first instar larvae to normal adults on *Ambrosiella* species from both major clades. An average of 40.5% of beetles from each gallery survived to the adult stage. There was no significant difference in beetle survival between the typical symbiont, *A. xylebori*, and either alternative symbionts; *A. grossmanniae* (binomial GLM; $z = 1.539$, $p = 0.12$) and *A. beaveri* ($z = 1.291$, $p = 0.20$).

(d) Mycangium assay

The mycangium of emerging *X. compactus* adults was significantly more likely to be full when the beetle was incubated with an *Ambrosiella* fungus from the *xylebori* clade (the same clade as their typical symbiont, *A. xylebori*) than when reared on *Ambrosiella* from *beaveri* clade fungi (binomial GLMM, $z = -2.825$, $p = 0.004$; figure 3*b*). At the species level, emergent females reared with the *beaveri* clade fungus *A. nakashimae* were significantly less likely to have a full mycangium ($z = -2.025$, $p = 0.043$), while all other species were not different from the reference group. Once a fungus was established in the mycangium of *X. compactus*, there were no significant differences among *Ambrosiella* species in the number of propagules produced, except for *A. roeperi* which was significantly lower than all other *Ambrosiella* species tested ($p < 0.05$ for all pairwise comparisons that included *A. roeperi*; Tukey's HSD *post hoc* test; figure 3*b*). The significantly lower propagule production of *A. roeperi in vitro* is consistent with previous experiments that showed variable and often low propagule production of this species within the mycangium of its typical vector *X. crassiusculus* [54]. Excluding *A. roeperi*, mycangial growth under our experimental conditions was comparable to natural conditions (i.e. the CFU estimates for all other fungal treatments were not significantly different from wild-caught dispersing *X. compactus* females and were similar to previous surveys of *X. compactus* caught in flight [50]).

4. Discussion

The diverse and globally distributed mutualism between beetles in the *Xylosandrus* complex and *Ambrosiella* fungi stemmed from a single evolutionary origin. Throughout the co-diversification of the *Xylosandrus* complex and *Ambrosiella*, this mutualism has not been species-specific, nor has it been entirely promiscuous. Instead, this association displays coarse-scale phylogenetic fidelity, such that each genus of beetle (except *Diuncus*) has remained exclusively associated with only one of the major clades of *Ambrosiella* fungi. However, the loss of co-phylogenetic signal at finer phylogenetic scales indicates that *Xylosandrus* beetles have frequently switched fungi within the *xylebori* and *beaveri* fungal clades, but switches between these clades have occurred much less frequently, perhaps only before the two *Ambrosiella* lineages had diverged. We can infer only one such switch, in the ancestor of *X. brevis* and *X. amputatus* after the divergence of *X. crassiusculus*. Phylogenetic analysis placed *Diuncus*

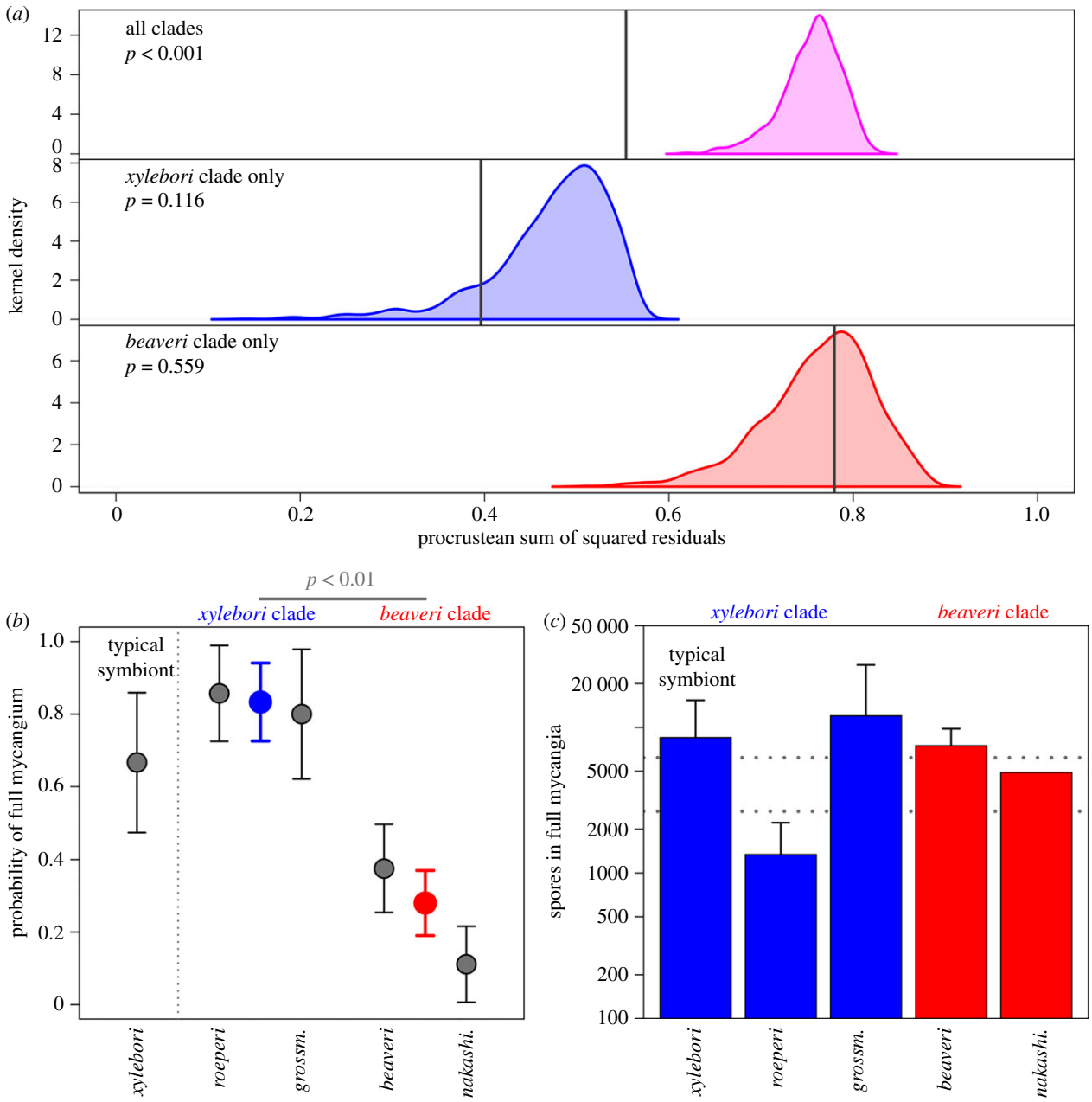

Figure 3. Mycangium selectivity explains coarse phylogenetic congruence in *Xylosandrus* and *Ambrosiella*. (*a*) Ambrosia beetle lineages have maintained fidelity to either fungal clade but have been promiscuous within those clades. Permutations-based null model hypothesis test of phylogenetic congruence based on pairwise distances: coloured areas show null distributions of Procrustean sums of squares (higher values indicate less congruence) for 1000 permutations of the association matrix. Grey vertical lines show observed sums of squares. The observed sums of squares for all associations were lower than all permutations of the association matrix, indicating highly significant congruence. There was no significant congruence when the analysis was restricted to only association involving either the *xylebori* clade fungi or the *beaveri* clade. (*b*) In symbiont switching experiments, ambrosia fungi from the alternative *beaveri* clade were significantly less likely to be taken up by the mycangium of *X. compactus* than the typical symbiont (*A. xylebori*) and others in the *xylebori* clade. Model estimates for each clade (excluding typical symbiont) are shown as coloured symbols. (*c*) Experimental subjects had fungal spore loads similar to wild collected beetles for all *Ambrosiella* species except *A. roeperi*. Error bars represent 95% CI. Horizontal dotted lines show 95% CI for estimates from wild *X. compactus* caught while flying. (Online version in colour.)

within the *Xylosandrus* complex, confirming the hypothesis that the mycangium was secondarily lost when this lineage began stealing the fungal cultivars of other ambrosia beetles [35].

We provide the first experimental demonstration that the ambrosia beetle mycangium is selective among congeneric lineages of ambrosia fungi and that selectivity explains coevolutionary patterns between ambrosia beetles and ambrosia fungi. Beetles incubated with their typical symbiont or alternative symbionts from the same clade (i.e. the *xylebori* clade) were approximately three times more likely to emerge from their galleries with mycangia bearing fungal propagules than beetles incubated with fungi from an alternative clade. Therefore, the mycangium in this system is not strictly species-specific in selecting fungal symbionts, nor does it discriminate completely at any level within *Ambrosiella*, but instead it makes acquisition of novel fungal partners increasingly unlikely with increasing phylogenetic distance from the typical symbiont. In doing so, the mycangium reinforces fidelity at coarse co-phylogenetic scales, while allowing promiscuity among closely related fungal symbionts. The specific mechanisms that drive selectivity in the mycangium are still unknown but could include negative feedbacks such as chemical or immunological exclusion of non-target fungi

and positive feedbacks such as nutritional sources that can only be used by target fungi, and may involve host behaviours that make the mycangium more or less accessible to various fungi. Future theoretical and empirical research is needed to determine if the maintenance of this intermediate degree of fidelity that is based on phylogenetic relatedness is an adaptive trait and a stabilizing mechanism in the evolution of mutualisms.

Other proposed mechanisms for fidelity between ambrosia beetles and their fungi were not supported. Though transmission of ambrosia fungi appears vertical in effect [55], it is actually transmitted indirectly from parent to offspring. Ambrosia beetles do not obtain their symbionts until they have completed development to the adult stage when the mycangium develops, and the fungal symbiont is acquired from the gallery, not through direct contact with the mother [54]. This transmission mode provides ample opportunity for horizontal transmission because fungal symbionts extend throughout the wood and invade neighbouring galleries [12,28,56]. We found evidence of frequent horizontal symbiont switching at fine phylogenetic scales as loss of phylogenetic congruence within major clades of symbionts. Our result is congruent with a recent co-phylogenetic study of *Euwallacea* ambrosia beetles and ambrosial *Fusarium* [6]. Therefore, strict vertical transmission cannot explain co-phylogenetic congruence in ambrosia beetles. Geographic isolation also cannot explain co-phylogenetic congruence. Many beetles in the *Xylosandrus* complex have extensive geographic ranges, and the co-occurrence of several to many species in dead trees of tropical and sub-tropical forests is typical [57]. It has been recently hypothesized that *Xylosandrus* beetles maintain fidelity to their fungi by seeking wood substrates that are rich in ethanol which provides a selectively harsh environment that screens out antagonistic non-ambrosial fungi such as *Penicillium* and *Aspergillus* [58]. However, all species in the *Xylosandrus* complex, as well as many other ambrosia beetle groups (e.g. *Ambrosiodmus*, *Corthylus*, *Euwallacea*, *Xyleborus* and *Xyleborinus*), are attracted to the ethanol released by stressed or dying trees. Owing to this shared attraction, dying trees in the tropics and subtropics are typically perforated by intermingled galleries, each inoculated with various species of ambrosia fungi. Consequently, ethanol screening cannot explain the co-phylogenetic congruence between *Xylosandrus* and *Ambrosiella* clades over evolutionary time shown here, or broader clade-level fidelity among independently derived ambrosia systems [59].

Nutritional specificity also does not explain fidelity in ambrosia symbioses. Larval *X. compactus* placed in experimental galleries with alternative fungal symbionts from either *Ambrosiella* clade began grazing on fungal spores within minutes (electronic supplementary material, video supplement V1). *Xylosandrus compactus* were successfully raised from eggs to adults on fungi from both major clades of *Ambrosiella*, and there were no significant differences among the tested *Ambrosiella* species. *Xylosandrus compactus* and its mycangial symbiont have been the focus of extensive study [23,50,60,61], and in every case the symbionts isolated from the mycangium were identified as *A. xylebori*. Thus, our experimental results demonstrate that even a beetle which displays very high fidelity for its fungal symbionts in nature, can consume and develop normally on alternative fungi, and thus symbiont specificity cannot be reliably inferred from even extensive field observations of host/symbiont associations alone.

Observations from *Diuncus* confirmed several important points. First, the parasitic lifestyle of *Diuncus* demonstrates that the transmission of ambrosia fungi is not necessarily vertical because every generation of *Diuncus* larvae is fed by horizontally transmitted ambrosia fungi that infiltrate their galleries through the xylem from neighbouring galleries [35]. Second, *Diuncus* was observed parasitizing a diversity of beetles in the *Xylosandrus* complex, including farmers of either major *Ambrosiella* clade, confirming that beetles in the *Xylosandrus* complex are not nutritionally limited to specific species or broader clades of *Ambrosiella*. Finally, the loss of mycangia in *Diuncus* was commensurate with a unique lack of fidelity to either *Ambrosiella* clade, further implicating the mycangium as a mechanism for coarse-scale fidelity among species within the *Xylosandrus* complex and *Ambrosiella* fungi.

While selectivity of the ambrosia beetle mycangium maintains fidelity at coarse phylogenetic scales, geography, ecology and climatic differences have stronger influences on symbiotic associations at finer scales. Historical shifts in distribution may have allowed co-speciation, which may reflect finer patterns within clades. Within the *Ambrosiella xylebori* clade, a monophyletic fungal clade is associated with a paraphyletic group of beetles that share biogeographic histories. The beetles *Anisandrus dispar*, *A. sayi*, *A. maiche* and *X. germanus* represent different genera, but are unusual among the *Xylosandrus* complex because they are all found in temperate areas as opposed to the tropics. Interestingly, these beetles are together associated with one monophyletic clade of *Ambrosiella*. We speculate that this clade of fungi may be particularly cold hardy and acquired separately by each temperate beetle lineage as they progressed northward from the tropics. There is also a clade of Australasian *Xylosandrus* (*X. russulus*, *X. monteithi* and *X. rotundicollis*) in which the symbionts are entirely unknown and may have evolved independently with their own *Ambrosiella* lineage.

The results reported here have important practical implications for species introductions, global silviculture and forest health. Anthropogenic introductions are increasingly bringing together novel combinations of insects and fungal plant pathogens, with potentially massive negative impacts. While most introduced ambrosia beetles have had little to no negative impact, a few have been devastating [37,62]. For example, a single introduction of the red bay ambrosia beetle (*Xyleborus glabratus*) and its symbiont *Raffaelea lauricola*, the causal agent of laurel wilt disease, has caused the death of more than 300 million lauraceous trees and altered forest community structure throughout the southeastern United States [63,64]. While attacks from the red bay ambrosia beetle are mostly limited to the red bay (*Persea borbonia*), horizontal transmission of the pathogen to alternative ambrosia beetle species with broader host tree preferences is negatively impacting avocado (*Persea americana*) industries [62]. Our results demonstrate that knowledge of the co-phylogenetic relationships of ambrosia beetles and their symbionts determines their risk level and can inform future invasive species management decisions. Our capacity to identify which invading beetles are likely to serve as alternative vectors for introduced pathogenic fungi, and which ones are likely to remain harmless, allows agencies to focus their limited resources on monitoring and controlling the most relevant species.

Data accessibility. The data supporting this article are available in the Dryad Digital Repository at: http://dx.doi.org/10.5061/dryad.

4bm536k [65]. All sequence data are available on GenBank (see electronic supplementary material, tables S1 and S2 for accession numbers).

Authors' contributions. J.S., A.J.J., M.A.J., C.C.B., Y.L. and J.H. collected specimens and data, assisted in the intellectual development of the project and contributed to final manuscript preparation. J.S. and A.J.J. analyzed data, produced figures and prepared initial manuscript draft.

Competing interests. We declare we have no competing interests.

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