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REPEATED EVOLUTION OF CROP THEFT IN FUNGUS-FARMING AMBROSIA BEETLES

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Ambrosia beetles, dominant wood degraders in the tropics, create tunnels in dead trees and employ gardens of symbiotic fungi to extract nutrients from wood. Specificity of the beetle–fungus relationship has rarely been examined, and simple vertical transmission of a specific fungal cultivar by each beetle species is often assumed in literature. We report repeated evolution of fungal crop stealing, termed mycocleptism, among ambrosia beetles. The mycocleptic species seek brood galleries of other species, and exploit their established fungal gardens by tunneling through the ambient mycelium-laden wood. Instead of carrying their own fungal sybmbionts, mycocleptae depend on adopting the fungal assemblages of their host species, as shown by an analysis of fungal DNA from beetle galleries. The evidence for widespread horizontal exchange of fungi between beetles challenges the traditional concept of ambrosia fungi as species-specific symbionts. Fungus stealing appears to be an evolutionarily successful strategy. It evolved independently in several beetle clades, two of which have radiated, and at least one case was accompanied by a loss of the beetles' fungus-transporting organs. We demonstrate this using the first robust phylogeny of one of the world's largest group of ambrosia beetles, Xyleborini.

KEY WORDS: Evolution of parasitism, horizontal transfer, mycangia, symbiont specificity.

One of the most remarkable examples of symbiosis is fungus farming, which has evolved independently in termites, ants, wood wasps, and ambrosia beetles (Mueller et al. 2005). In most of these associations, the fungal partner provides an enzymatic apparatus for extracting nutrients from abundant but hard-to-digest substrates such as wood or leaves. The animal partner provides suitable substrate and protection (Martin 1992). One of the most variable aspects of these associations is the mechanism by which the animal–fungus connection is sustained through generations. For example, the most highly evolved fungus-growing ants and ambrosia beetles possess organs for secure transmission of symbiont spores during dispersal and hence across generations. On the other hand, some fungus-growing termites acquire symbionts from the environment anew every generation. Between these two extremes lies a spectrum of strategies found in fungus farming ants, wood wasps, and beetles. Most of these species actively transport their symbionts, but are capable of, or even dependent on supplementing their fungus gardens by obtaining strains from the environment.

In this study, we document a newly discovered strategy of acquiring symbiotic fungus in ambrosia beetle—symbiont theft. We term the strategy "mycocleptism." The capacity for stealing

[4]Both authors contributed to designing the project and writing the paper. JH performed the research and analyzed the data.
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inoculum of symbiotic fungi from gardens of sympatric or related species has been observed in ants. Some genera, such as *Cyphomyrmex*, steal fungal symbionts from different nests as a response to an accidental loss of their own fungus garden (Adams et al. 2000a). In other ants, such as *Megalomyrmex* or *Pseudoatta*, social parasitism evolved into a fungus theft as an obligate foraging strategy (Adams et al. 2000b; Sumner et al. 2004). Here, we report for the first time similar dependence on symbiont crop theft in mycocleptic ambrosia beetles, where it also evolved several times, and has become the main foraging strategy of at least 16 species.

Ambrosia beetles create tunnels (galleries) in dead wood, where they feed and develop on symbiotic wood-decay fungi. This fungus is transported from the natal colony in specialized pouches called mycangia. Ambrosia beetles are a true showcase of the success of the fungus farming strategy: they dominate beetle communities in tropical forests (Noriega et al. 2007), are among the most frequent invasive species worldwide (Haack 2006; Rabaglia 2006), and the damage they cause to forest and timber industry parallels that by the notorious tree-killing bark beetles (Orbay et al. 1994; Fraedrich et al. 2008). Ambrosia beetles and their fungi are an important force in degradation of woody biomass, as a single dead tree can be colonized by thousands of beetles (Hulcr et al., 2007a). Ambrosia beetles are promising for the study of symbiotic evolution—they are the only fungus-growing insects to have evolved the strategy multiple times, and therefore provide an unparalleled framework for comparative studies (Farrell et al. 2001).

The mechanisms behind the beetle–fungus association remain elusive. Most ambrosia beetles possess mycangia, invaginated cuticular pouches specialized for transporting fungal spores. These are usually thought to transport species-specific fungal symbionts (Mueller et al. 2005). More detailed analyses suggest that beetles may maintain a stable community of symbiotic fungi (Kuhnholz 2004; Harrington 2005), or that the beetles can coexist with and develop on a number of interchangeable fungi recruited from the environment (Batra 1966).

Our recent extensive observations in the field suggested that a number of tropical ambrosia beetle species engage in a novel foraging strategy. These beetles extended the occasional acquisition of fungal symbionts from the environment to a dependency on obtaining the fungal crop from other ambrosia beetles—mycocleptism. Mycocleptic species seek established brood galleries of "host" beetle species, and create their own galleries adjacent to the host tunnels (Fig. 1). Mycelia originating from the established garden of the host species are interrupted and diverted to create fruiting structures in the mycocleptes' galleries.

In New Guinea and Borneo, we tested several predictions of the hypothesis that mycocleptism is a repeatedly evolved and ecologically successful foraging strategy: (1) mycocleptae acquire fungi from their host beetles, (2) mycocleptic lineages lack structures for symbiont transport, (3) mycocleptae target the immediate vicinity of the providers' galleries, instead of creating independent galleries as other ambrosia beetles, and (4) this strategy evolved repeatedly in independent lineages of ambrosia beetles.

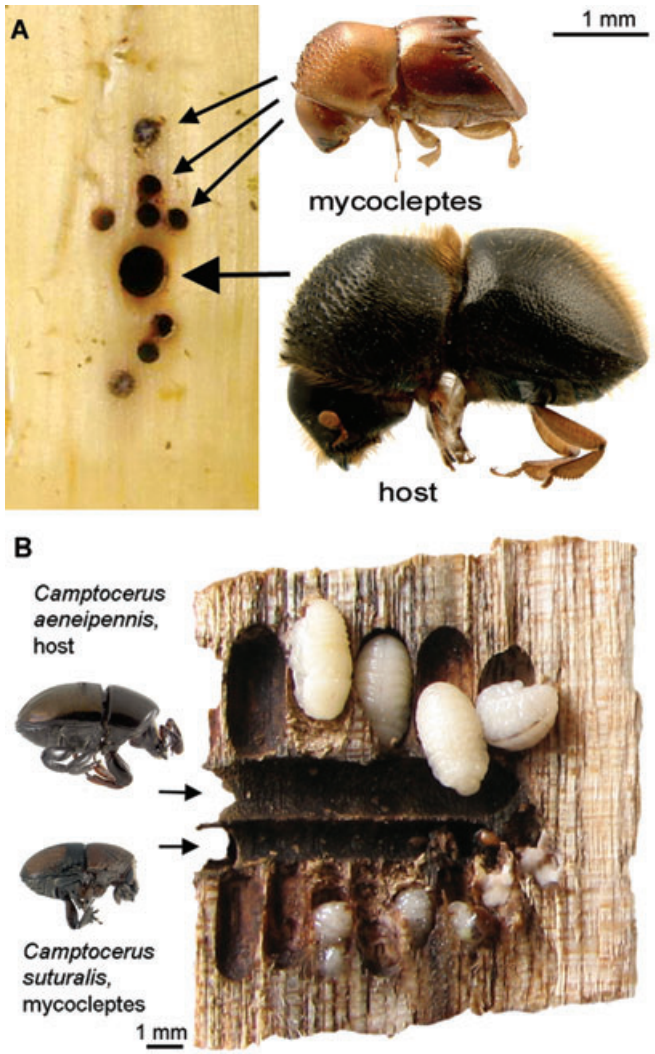

Figure 1. **Mycocleptism in vivo. (A) Entrances of multiple small galleries of mycocleptic *Diuncus duodecimspinatus* concentrated around the single large entrance of the provider species *Hadrodemius globus*. Branch of *Ficus* sp., Papua New Guinea, photos: JH B: Detrimental effect of mycocleptae on their host beetles. Gallery and larval chambers of *Camptocerus aeneipennis* (Guyana), of which the bottom half was destroyed and replaced by a gallery and brood of mycocleptic *Camptocerus suturalis* (the lower, smaller gallery and larval chambers). In healthy galleries of *C. aeneipennis*, larval chambers line both sides of the maternal tunnel. *Camptocerus* photo: Sarah M. Smith, Michigan State University.**

Materials and Methods

Beetles and their associated fungi were collected as a part of larger sampling project (~44,000 scolytine samples, Hulcr et al. 2007a) in Papua New Guinea (Madang, 200 m, 145°40′E, 5°14′S; Popondetta, 220 m, 148°12′E, 8°48′S; Kanga, 500 m, 147°38′E, 8°46′S, Mu, 1800 m, 145°02′E, 6°05′S), and in Sabah, Malaysia (Danum Valley, 117°50′E, 4°58′N).

FUNGUS SHARING

Twenty nine galleries of mycocleptae and their host beetles collected into separate sterile vials were chosen for fungal DNA extraction (one complete gallery per individual beetle). The samples were preserved either in 100% EtOH and frozen, or stored in mineral oil at 4°C to preserve viable fungi. Fungal tissue was scraped from walls of the galleries, the mixture of wood and fungal tissue was ground in Qiagen ATL buffer, and the genomic DNA extracted using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Fungal rDNA of the ITS I region (between SSU RNA and 5.8S RNA) was amplified using primers ITS1F (forward, optimized for fungi [Gardes and Bruns 1993]) and ITS2 (reverse, general [White et al. 1990]). The PCR product was cloned into chemically competent *Escherichia coli* cells in Invitrogen TOPO-TA cloning kit. The ITS insert was sequenced from 24 successfully transformed colonies from each gallery. Fungal sequences were identified using NCBI BLAST (blastn) and deposited in GenBank (FJ807989– FJ808073).

To corroborate culture-independent data, fungal inhabitants of several gallery samples from the same trees and beetle species were also cultured (Prikryl et al. 2010). To isolate all fungi or to enrich for scolytine-associated Ophiostomatales, yeast-malt extract agar was used with or without cycloheximide, respectively (Harrington 1981).

The analysis of fungal communities was based on a matrix of pairwise Sørensen similarities between all beetle galleries. The similarity was derived from presence or absence of fungal strains as identified by their ITS1 sequence. No attempt was made to measure phylogenetic distance between the fungal gardens, as the ITS1 sequence was short and partly unalignable. We calculated the distribution of similarity of fungal communities between individual galleries of unassociated heterospecific beetles (Sørensen pairwise similarity, $S_{mean} = 0.184$, $n = 346$), to derive baseline measure of lowest, or background similarity. We calculated the same distribution of similarity between gardens of conspecific (but not mycocleptic) beetles ($S_{mean} = 0.489$, $n = 9$), to derive the highest hypothetical similarity in the ambrosia community. Confidence intervals (95%) around the means of these distributions were estimated by bootstrapping (1000 random samples with replacement). These intervals were used to test two assumptions of the hypothesis that mycocleptae steal fungi from their hosts. First, we tested whether the similarity of fungal gardens of conspecific mycocleptae associated with different hosts approaches the similarity among unrelated heterospecifics. Then we tested whether the similarity of gardens of heterospecific mycocleptae associated with the same hosts equal the similarity among general conspecifics.

MYCANGIA

We explored the presence or absence of mycangia in one mycocleptic genus (10 *Diuncus* spp. examined), nonmycocleptic species with known mesothoracic mycangia (all five genera in the respective clade examined), and multiple species from genera with mandibular mycangia (*Amasa, Euwallacea, Xyleborus* spp.)(Fig. 1, Table S1). The large mesothoracic mycangia are easily visible in beetles dissected under a stereo-microscope. For visualizing mandibular mycangia, beetle heads were fixed in 96% ethanol, immersed in 30% hydrogen peroxide for 24 h, and embedded in paraffin. The preparates were sectioned on a rotary microtome at 5 μm and stained with hematoxylin and eosin.

PHYLOGENY

The phylogeny of Xyleborini was reconstructed using partial sequences of the mitochondrial gene COI, and nuclear genes ribosomal 28S, ArgK, EF1-alpha, and CAD (=*rudimentary*), for a total of 3925 bp, from 62 species of Xyleborini. DNA isolation, PCR and sequencing followed the same protocol as for the fungal DNA, with previously published beetle-specific primer sequences (Jordal 2002, 2007; Hebert et al. 2003; Jordal et al. 2007). Two species from the closely related genus *Coccotrypes* (Dryocoetini) (Jordal et al. 2000) were used as outgroups. DNA sequences were deposited in GenBank (see Supporting information). Sequences were aligned for each gene separately using MUSCLE (Edgar 2004). The program MrModeltest was used to select the appropriate models of evolution and the priors (Nylander 2004). The following partitioning scheme and sequence evolution models were used for a Bayesian phylogenetic analysis: (1) 28S (GTR + I + G, unalignable parts excluded), (2) First positions of nuclear protein-coding genes (GTR + I + G), (3) Second positions of nuclear protein-coding genes (GTR + I + G), (4) Third positions of nuclear protein-coding genes (GTR + I + G), (5) COI, first positions (SYM + I + G), (6) COI, second positions (F18 + I + G), (7) COI, Third positions (GTR + I + G). The phylogeny was inferred using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), after 2 million generations of two simultaneous runs and four search chains, sampled each 1000 generations. The trees from the first 47% generations, when the average standard deviation of split frequencies between the two runs dropped was below 0.01, were discarded as burn-in. The probability of the monophyly of all mycocleptic species was calculated as a ratio of the MCMC-sampled trees (after burn-in) in which this clade appears over all sampled

trees. For the complete phylogeny, see Supporting information (Fig. S1). The data matrix and resulting tree were deposited in TreeBase (submission ID number: 10518).

CO-OCCURRENCE

At one locality in Sabah, Malaysia, and one locality in Madang Province, Papua New Guinea, we selected 10 dead trees or large fallen branches colonized by known host beetle species, and sampled them exhaustively. Each gallery or a set of closely positioned galleries was excised from the tree and dissected by hand. Beetles from each gallery were stored in a separate vial and identified in the laboratory. We recorded whenever a putative mycocleptic species was found (1) in the direct vicinity of a provider species' gallery (within 0–10 mm), (2) in an independent gallery and alive, and (3) in an independent gallery and dead. The significance of differences between the categories was assessed with repeated measures analysis of variance (ANOVA) ($N = 92$, dft $= 2$, dfe $=$ 24, $F = 35.51$, $P < 0.0001$).

Results

To test whether mycocleptae indeed acquire fungi from galleries of their host beetles, instead of introducing their own fungal associates, we sequenced fungal DNA (rDNA ITS1 region) directly from the galleries of both the mycocleptae and their hosts, as well as from unassociated ambrosia beetle species occupying the same trees. From 29 galleries of 15 ambrosia beetle species (each gallery occupied by a single family), we isolated 52 unique fungal strains, one to eight species per gallery (Supporting Information, Table S2). The fungal community was dominated by *Ceratosystis* spp. and *Fusarium* aff. spp. The commonly reported ambrosia fungi of the genera *Ambrosiella* and *Raffaelea* were not detected. Their absence was confirmed by BLAST searches with our sequences, as well as by direct comparison of our sequences to those identified as *Ambrosiella* and *Raffaelea* in the NCBI database. To corroborate the culture-independent data, fungal inhabitants of the galleries were also cultured in vitro, which revealed a similar fungal community dominated by *Fusarium* and *Ceratocystis* (Prikryl et al. 2010).

The galleries of mycocleptae were mostly found to contain the same strains of fungi as their hosts' galleries, indicating that mycocleptae recurrently acquire fungi from their hosts (Fig. 2). The similarity of fungi between galleries of mycocleptae and their hosts was even slightly higher than the similarity among nonmycocleptic conspecifics ($\text{Sørensen}_{\text{mean}} = 0.588$, $n = 11$, vs. $S_{\text{mean}} = 0.489$, $n = 9$; note that the latter low value indicates that beetle-fungus associations are not strictly species specific). Consequently, fungal gardens of conspecific mycocleptic beetles are not similar if the same mycocleptae are parasitizing different

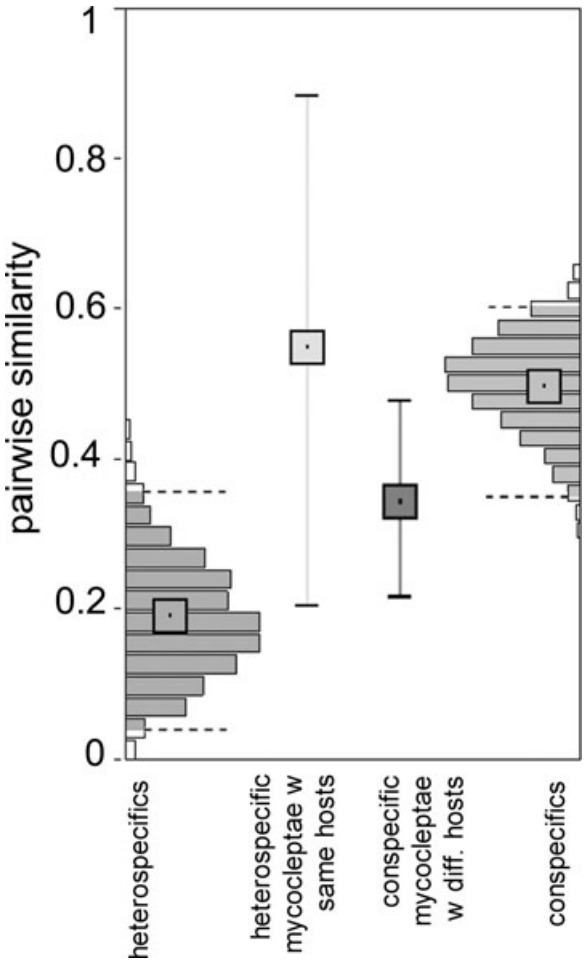

Figure 2. Pairwise similarity of fungal assemblages between combinations of mycocleptae, their hosts, and nonhosts. Fungal similarity among nonmycocleptic heterospecifics and conspecifics is given as mean, probability density of mean from bootstrapped datasets (1000× resampled), and 95% confidence intervals, allowing statistical comparison with similarities among mycocleptae. Pairwise similarities between mycocleptae and their hosts and between conspecific mycocleptae associated with different hosts are given as mean and ±SD.

host species ($S_{\text{mean}} = 0.365$, $n = 12$, $P = 0.005$), indicating that mycocleptae probably do not carry their own symbionts.

The hypothesis that mycocleptae do not possess their own means of transporting fungal inoculum—mycangia—was tested with *Diuncus*, the largest mycocleptic genus within the subtribe Xyleborini, which itself is one of largest groups of ambrosia beetles. Xyleborini have three types of mycangia: elytral, mandibular, and mesonotal (Fig. 3). Mapping mycangial evolution on a five-gene Bayesian phylogeny of Xyleborini suggests that mycangium type is a very conservative trait, because each type evolved only once. Mandibular mycangia appear to be ancestral in Xyleborini (Fig. 4, Table S1). Histological dissections of *Diuncus* spp. revealed absence of all three types of mycangia.

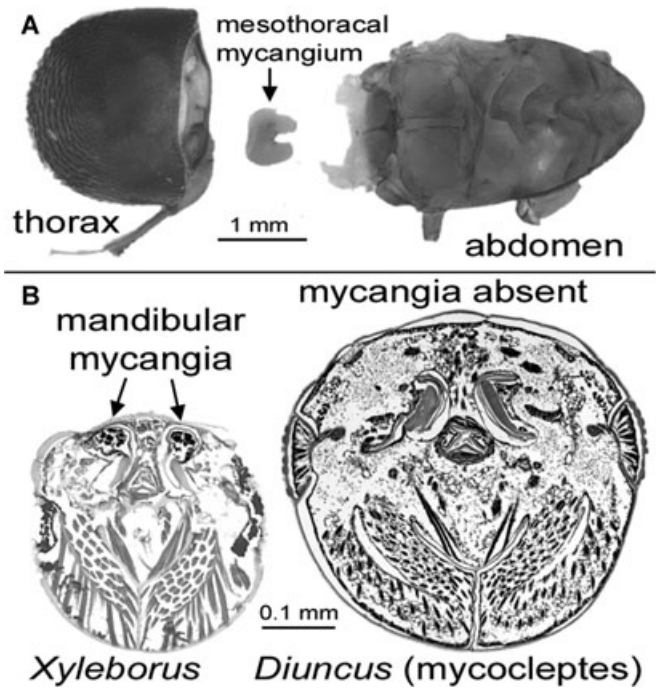

Figure 3. Mycangia. (A) Mesothoracic mycangium in dissected *Xylosandrus germanus*. This mycangium is absent in the related but mycocleptic genus *Diuncus*. (B) Cross-section of heads of *Xyleborus affinis* and the mycocleptic *Diuncus duodecimspinatus*, showing the ancestral mandibular mycangia in the former, and their absence in the latter species.

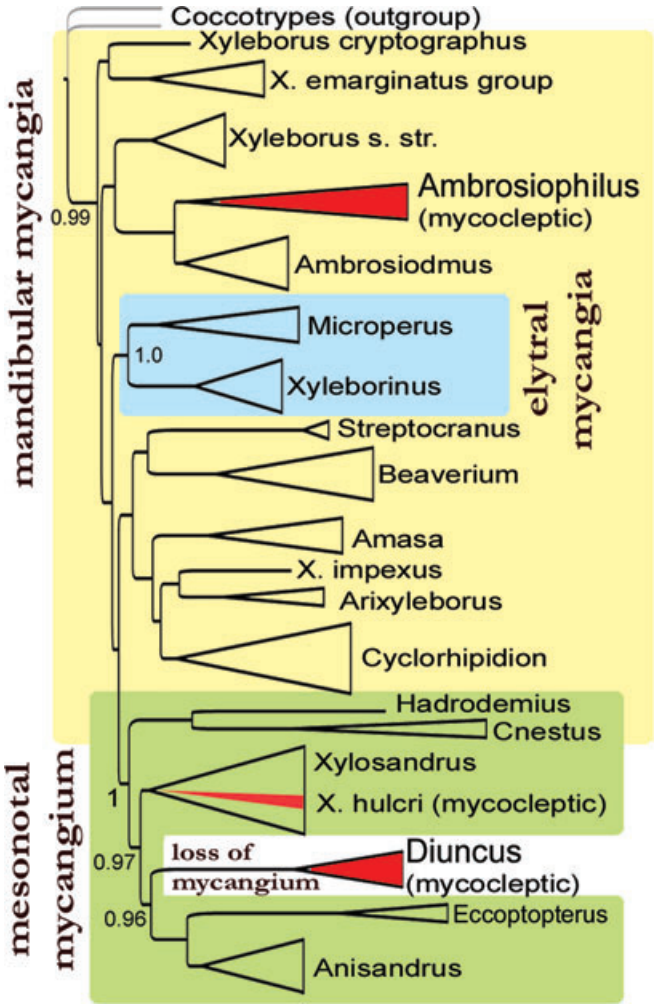

Figure 4. Bayesian five-gene phylogeny of Xyleborini showing the ancestral position of mandibular mycangia (yellow), separate origins of elytral (blue), and mesonotal mycangia (green), and the loss of mycangia in mycocleptic *Diuncus* (not examined in other mycocleptae). Red clades: confirmed mycocleptae. Clade width: number of analyzed species.

The phylogenetic position of the genus indicates that the mycangium has been secondarily lost (posterior probabilities of the three containing clades: 0.92, 0.92, and 0.99, see Supporting information). The phylogeny also indicates that the three confirmed instances of mycocleptism in Xyleborini, *Diuncus*, *Ambrosiophilus* and *Xylosandrus hulcri,* evolved independently. The posterior probability of monophyly of all mycocleptae is zero, as none of the Markov chain Monte Carlo sampled trees contained such clade.

The hypothesis that mycocleptae actively seek a provider species was tested using the patterns of co-occurrence of the associates in the rainforests of New Guinea and Borneo. Virtually all mycocleptae establish their galleries within 1 cm of host beetle galleries ($n = 85$, $P = 0.0001$). Species of *Diuncus* also occasionally create independent galleries (five individuals, 6%), separated from any potential host gallery; however, beetles were found to be dead in approximately half of these. In many instances, several mycocleptae were found associated with a single gallery of the host.

To date, 15 Paleotropical and one Neotropical ambrosia beetle species (five genera, Table 1) were confirmed mycocleptae, and were observed with 19 host beetle species (at least ten genera). In New Guinea, approximately 4% of known ambrosia beetle species are mycocleptae. Most mycocleptae display notable host specificity, although quantitative data are not available. For example, our records indicate that majority of mycocleptic *Ambrosiophilus* are routinely found associated with *Beaverium* spp., *Diuncus papatrae* is found almost exclusively with *Anisandrus ursa*, and *Diuncus duodecimspinatus* with *Hadrodemius globus*. In South America, the mycocleptic *Camptocerus suturalis* is reported almost exclusively from the vicinity of *Camptocerus aeneipennis* (S. M. Smith, pers comm.). Earlier records indicate that some of the same associations may occur throughout the Indo-Pacific region (Kalshoven 1960; Beaver 1976). Some mycocleptae, especially *Camptocerus suturalis*, have been observed to chew through their hosts' galleries, destroying a significant portion of the host's brood (Fig. 1).

Table 1. Confirmed records of mycocleptae and their hosts, by J H except when noted.

Taxonomic group	Genus	Species	Recorded hosts	Origin
		mogia	*Beaverium perplexus*	New Guinea
	Ambrosiophilus	*restrictus*	*Beaverium insulindicus, Beaverium latus, Beaverium sp. "aero", Beaverium sundaensis*	Sabah, New Guinea
		semicarinatus	*Beaverium insulindicus*	New Guinea
		sexdentatus	*Beaverium sundaensis*	New Guinea
		conidens	*Xylosandrus borneensis*	Sabah
		duodecimspinatus	*Eccoptopterus spinosus, Hadrodemius globus*	New Guinea
Xyleborini		*haberkorni*	*Eccoptopterus spinosus*	New Guinea
		justus	*Amasa resectus, Xylosandrus morigerus*	Sabah, New Guinea
	Diuncus	*mesoleiulus*	*Cyclorhipidion multipunctatus*	New Guinea
		niger	*Xylosandrus russulus*	New Guinea
		papatrae	*Anisandrus ursa, Xylosandrus crassiusculus, Xylosandrus russulus*	New Guinea
		quadrispinosulus	*Eccoptopterus spinosus*	New Guinea
		sp. "concave"	*Cnestus ater, Scolytoplatypus javanus*	Sabah
	Xylosandrus	*hulcri*	*Xylosandrus russulus*	New Guinea
Scolytini	*Camptocerus*	*suturalis*	*Camptocerus aeneipennis*	Guyana, Peru (S.M. Smith)
Platypodinae	*Crossotarsus*	*imitatrix*	*Crossotarsus longicornis*	New Guinea

Discussion

Although occasional symbiont theft has been known in ants, the frequency with which it occurs in ambrosia beetles as a principal foraging strategy is unprecedented. Fungal crop theft in ambrosia beetles appears to be an evolutionarily successful adaptation, as indicated by the multiple origins of the strategy followed by at least two radiations (*Diuncus* and *Ambrosiophilus*, Fig. 4).

Anecdotal observations of putative "commensalism" in ambrosia beetles have been previously published (Kalshoven 1960; Beaver 1976). Our observations suggest that the effect of mycocleptism on the host beetles varies from neutral to parasitic. Many mycocleptae inflict loss on their hosts, ranging from destroying the host's gallery (Fig. 1), to decreasing the amount of fungal matter available to the host's larvae, especially in cases of high density of mycocleptae (Fig. 1). For fungus-feeding scolytine larvae, symbiotic fungi are a limiting resource, directly affecting larval development (Ayres et al. 2000; Bleiker and Six 2007). We hypothesize that mycocleptae benefit from their behavior mostly by securing abundant fungal food, without the risk of their own garden failure.

An intriguing aspect of the mycocleptic strategy is the mechanism by which mycocleptae locate galleries of their host beetles. Spatial orientation and host location in scolytine beetles is a complex behavior integrating tree volatile chemicals and intraspecific pheromones (Wood 1982). Inbreeding ambrosia beetles such as Xyleborini have not been shown to use pheromones, and only non-specific dying tree odors are known to serve as attractants (Ranger et al. 2010). Discernment of a single gallery of an unrelated beetle by mycocleptae suggests unknown sensory mechanisms. It is possible that the attractant is fungus-derived, however, the limited specificity between fungi and beetle species in our dataset does not support this hypothesis.

Our analysis of ambrosia beetle galleries yielded a significant diversity of fungi. From occurrence data, it is difficult to ascertain which of the fungal species are nutritionally beneficial symbionts transmitted by the beetles, and which are auxiliary wood-associated mycobiota. However, the absence of *Ambrosiella* and *Raffaelea* suggest that these well-known ambrosia fungi might have been locally replaced by other taxa, such as *Ceratocystis* and *Fusarium*, which dominated both our DNA samples and live cultures. Both genera have been occasionally reported as scolytine associates (Norris 1979; Krokene and Solheim 1996; Morales-Ramos et al. 2000). Alternatively, because the genus *Ceratocystis* gave rise to several known species of the polyphyletic genus *Ambrosiella* (Alamouti et al. 2009), it is possible that our DNA isolates refer to ambrosial Microascales that are not yet represented in DNA databases (*Thielaviopsis* anamorph observed). Even though we cannot make conclusions about nutritional significance of these fungi, our data suggest that our contemporary understanding of the ambrosial community is incomplete, especially in regards to the rich tropical ambrosial ecosystem.

Xyleborini are one of the fastest radiating groups of scolytine beetles, having produced 1300 species in only 20 million years (Farrell et al. 2001). Yet until now, evolution of this megadiverse group remained unexplored, because their classification was based on a pre-phylogenetic typological taxonomy. Only recently have representatives of Xyleborini been included in molecular phylogenetic analyses (e.g., Jordal et al. 2000; Jordal 2002). The five-gene phylogeny presented in this article is the first that is both comprehensive and robust enough to allow hypothesis testing about Xyleborini evolution. Based on this phylogeny, we infer a single origin of each type of mycangium, and the multiple origins of mycocleptism. In the future, the phylogeny will be used in an ongoing reclassification of Xyleborini genera (Hulcr et al. 2007b).

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Supporting Information

The following supporting information is available for this article:

Figure S1. Detailed majority rule consensus tree from a five-gene Bayesian analysis of Xyleborini. Numbers on branches represent posterior probability.

Table S1. Fungal strains of rDNA ITS isolated from galleries of mycocleptic and free-living beetles and identified using NCBI BLAST.

Table S2. NCBI Genbank ID's of DNA sequences for the *Xyleborini ambrosia* beetle phylogeny.

Supporting Information may be found in the online version of this article.

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