Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic

***Cecropia* sp. trees – diversity and patterns**

Maximilian Nepel^{1,2}, Hermann Voglmayr¹, Rumsais Blatrix³, John T. Longino⁴, Konrad Fiedler¹, Jürg Schönenberger¹ and Veronika E. Mayer¹

¹ Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

² Department of Microbiology and Ecosystem Science, University of Vienna, Althanstraße 14, A-1090 Vienna, Austria

³ CEFU UMR 5175, CNRS – Université de Montpellier – Université Paul Valéry Montpellier – EPHE, 1919 route de Mende, 34293 Montpellier Cedex 5, France

⁴ Department of Biology, 257 S 1400 E, University of Utah, Salt Lake City, UT 84112, USA

Corresponding author:

Veronika Mayer

Email: veronika.mayer@univie.ac.at

Tel: +43 1 4277 54081

Fax: +43 1 4277 9541

Abstract. Five *Cecropia* tree species occupied by four *Azteca* ant species from Costa Rica and French Guiana were investigated to assess the diversity and host specificity of chaetothyrialean fungal symbionts. The ITS rDNA region of the symbiotic fungi was sequenced either from pure culture isolation, or from environmental samples obtained from ant colonies nesting in hollow stems of the *Cecropia* host plants. The investigation revealed six closely related OTUs of *Chaetothyriales*. Neither the four *Azteca* species nor the six fungal OTUs were associated with specific *Cecropia* species. In contrast, ants and fungi showed an association. *Azteca alfari* was associated with a particular OTU, and often contained only one. *Azteca coeruleipennis*, *A. constructor* and *A. xanthochroa* were associated with a different set of OTUs and often had multiple OTUs within colonies. Possible reasons for these differences and the role of the fungi for the *Azteca-Cecropia* symbiosis are discussed.

Key words: *Azteca* spp., *Cecropia* spp., *Chaetothyriales*, symbioses, ant-plant interactions

Introduction

Species interactions, and especially those that are mutually beneficial, are key factors in ecosystem function (Danovaro et al., 2008). Although mutualistic interactions are among the most fundamental in the adaptation and survival of species (Kiers et al., 2010), many mutualisms remain overlooked, especially those that involve microorganisms. Moreover, most mutualisms were first studied as bipartite interactions, but turned out to comprise complex networks of multiple species interactions (Caldera et al., 2009; Vazquez et al., 2009). Classic examples of such complex mutualisms are many cases of ant-plant symbioses (e.g. Chomicki *et al.* 2015).

True ant-plants (or “myrmecophytes”) provide ants with nesting cavities called domatia, which may be formed from leaf pouches, hollow stems, petioles, or stipular thorns (Davidson and McKey, 1993). In return, the ant associates protect their host plant against herbivores, pathogens and competing vegetation (Letourneau, 1998; Suarez et al., 1998; Rosumek et al., 2009). Nutritional mutualisms may also occur, in which ant waste is deposited inside domatia and nutrients are subsequently transferred to the host plant (Rico-Gray and Oliveira, 2007). It has been known for a long time that fungi occur in domatia of various ant-plants (Miehe, 1911; Bailey, 1920; Bailey, 1922; Bequaert, 1922; Docters van Leeuwen, 1929; Janzen, 1972; Huxley, 1978; Schremmer, 1984), but only recently have functional aspects of the interaction been investigated and their mutualistic nature been demonstrated (Blatrix et al., 2012; Defosse et al., 2009; Defosse et al., 2011). Also, many ants fabricate carton, a cardboard-like material, from masticated plant fibres, and they use it to build nests and galleries. Fungi have been found intimately associated with this carton (Dejean et al., 2005; Mayer and Voglmayr, 2009; Lauth et al., 2011; Leroy et al., 2011; Nepel et al., 2014). Recent investigations showed that fungi in the domatia of myrmecophytic plants, as well as in ant carton, mostly belong to the order Chaetothyriales in the Ascomycota, and, to a lesser extent,

Capnodiales (Schlick-Steiner et al., 2008; Mayer and Voglmayr, 2009; Ruiz-Gonzalez et al., 2011; Voglmayr et al., 2011). Especially within the domatia, fungi belong mostly to one chaetothyrialean clade, the so-called domatia symbiont clade (Voglmayr et al., 2011).

In the present study we focus on the occurrence of chaetothyrialean fungi in five species of the Neotropical plant genus *Cecropia* (Urticaceae) occupied by four species of *Azteca* (Formicidae: Dolichoderinae). *Cecropia* offers food to the ants in the form of glycogen rich food bodies (Müllerian bodies) produced on a mat of dense trichomes at the base of the leaf petiole and pearl bodies on the upper leaf side. They also provide nesting space in the hollow internodes (Bischof et al., 2013; Janzen, 1969; Müller, 1876, 1880; Rickson, 1971). The hollow internodes are often subdivided into functional units by ant-built carton structures (Fig. 1F). Overall, *Cecropia-Azteca* associations involve 44 myrmecophytic *Cecropia* and 13 associated *Azteca* species (Longino, 1991; Berg and Rosseli, 2005). The internodes also regularly contain small mounds of brown, sticky, bran-like material which have been observed by Müller (1880, described as "Klumpen") and Longino (1991). They are known to contain abundant nematodes and dipteran larvae, but here we show that they consist predominantly of fungi, and thus we call them "fungal patches." We investigated the fungi found at two different sites in Costa Rica and one site in French Guiana. First, the fungi were taxonomically classified by cultivation-dependent and cultivation-independent molecular methods. Second, we analysed whether the Chaetothyriales found in the domatia are specific to plant species, ant species, or geographical region. And third, in colonies where ant-made carton structures in domatia could be sampled as well, we explored whether the fungi are restricted to the patches or whether they also occur on the carton structures in the domatia.

Material and Methods

Study sites and sampling method

Study sites: We collected domatia from ant colonized *Cecropia* plants from two distinct locations in Costa Rica. Thirteen *Cecropia* trees occupied by *Azteca* were sampled in a lowland forest in the south-west of Costa Rica, near the tropical research facility La Gamba (N08°42'03'', W083°12'06'', 70m asl), in 2011 and 2012. Fourteen *Azteca*-inhabited trees were sampled in Monteverde, Costa Rica along the road from the town of Guacimál (N10°12'57'', W084°50'46'') to Santa Elena (N10°19'12'', W084°49'30'') (345 – 1448 m asl) in 2013 (Table S1). In 2009 we also collected fungal patches from one *C. palmata* (N5°32'25'', W53°25'20'') and one *C. obtusa* (N5°08'29'', W52°42'52'') growing along the coast of French Guiana and occupied by *A. alfari*.

Collection method: To take environmental samples, internodes which were hosting established ant colonies of the genus *Azteca* were cut open and sampled by collecting ant queens, workers, fungal patches and carton structures if present. Carton and fungal patches were collected separately to avoid cross-contamination of hyphae. For the analyses presented here we took only patches and carton from established ant colonies, defined by the occurrence of numerous workers and brood. From each colony we took at least three replicates from different parts of the *Cecropia* stem. In total we took environmental samples from 27 established *Azteca* colonies, 11 from the Costa Rican Pacific lowlands (La Gamba), 13 from the Costa Rican highlands near Monteverde cloud forest, and 2 along the coast of French Guiana (Table S1, supplementary information). *Azteca* ants were stored in 96 % and 70 % alcohol for later identification, whereas all patch and carton (if present) samples from the domatia were put into plastic vials, closed by a pellet of air permeable paper tissue, and dried using silica gel. Additionally, fungi were isolated for pure culture from three colonies (one *A. alfari*, one *A. constructor*, one *A. xanthochroa*).

Species identification: The *Cecropia* species were identified by flowers and fruits, or by leaf characteristics if flowers or fruits were absent, using Berg and Franco Rosselli (2005). Leaf characteristics can be rather variable in *Cecropia* and in case of uncertainty they are listed as “cf.” in Table S1. Molecular identification tools for the genus turned out to be difficult to apply because sequence variation among *Cecropia* species is extremely low (Treiber et al., 2016). Herbarium specimens and leaf samples in silica gel of all sampled trees are deposited at the herbarium of the University of Vienna (WU).

Azteca species were identified by J. Longino. If only workers were found, a DNA barcode was used for determination (see below).

The morphology of fungal hyphae in domatia was investigated using light microscopy as previously described (Nepel et al., 2014). Transmission electron microscopy was performed at the University of Montpellier with a Philips CM10 transmission electron microscope as previously described (Defosse et al., 2009).

Sample processing (Pure culture isolation, DNA extraction)

Pure culture isolation: The fungal patches were taken off the inner wall of the internodes, placed in a drop of sterile water and the hyphae carefully separated from each other. Conidiophores and hyphal fragments were transferred onto 2 % malt extract agar plates (MEA) supplemented with antibiotics (200 mg/l penicillin G and 200 mg/l streptomycin sulfate Sigma-Aldrich, St. Louis, MO) and checked for contaminants every 2h during the day for several days (for more details see Voglmayr et al., 2011 and Nepel et al., 2014). Fast growing contaminant fungi (for example *Fusarium*, *Cladosporium*, *Penicillium*) were cut out. Contamination-free growing Chaetothyriales hyphae were transferred onto new agar plates and grown at room temperature for several weeks, as they are very slow-growing in pure culture. For DNA extraction, sections of approximately 25 mm² were taken from mycelia on pure-culture agar plates, stored in 2 ml reaction tubes at -20 °C and freeze-dried overnight.

Then they were ground with five glass beads (3 mm diameter) for 10 min at 30 Hz in an MM 400 mixer mill (Retsch, Germany), after which DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Environmental samples: Environmental fungal patches and carton samples which had been silica gel dried in the field were directly ground and extracted in the same way as the pure culture mycelia. From each ant colony and host-tree at least three replicates of patch and, if present, carton samples were processed in this way.

Ant workers: In cases where no ant queens were found, we took two to three alcohol preserved worker ants, dried them and extracted DNA as described above for fungal samples.

PCR and sequencing

Fungal pure cultures: The DNA of pure cultures was amplified with the primers V9G (De Hoog and Gerrits van den Ende, 1998) and LR5 (Vilgalys and Hester, 1990) to obtain an rDNA gene fragment containing the partial small subunit (SSU), complete ITS1-5.8S-ITS2 (ITS), and the D1-D3 domains of the partial long subunit (LSU). Based on the resulting assignments of pure cultures and the study of Voglmayr et al. (2011) we designed two specific reverse primers for the “domatia symbiont clade” within the Chaetothyriales. The new primer chaeD-R (5' GCCCTACCGCAGTTCCA 3') is located in the LSU, whereas chaeDITS-R (5' AGTACGTGCTACAAGAGC 3') binds in the last 60bp of the ITS2 region.

Environmental samples of fungal patches and carton: For each fungal patch and carton sample PCR was conducted twice, once with primers V9G and chaeD-R and once with V9G and chaeDITS-R. We aimed to obtain the whole ITS region, and thus the chaeD-R primer, amplifying the complete ITS2, was preferred. In cases where this primer pair did not result in a single clear band in gel electrophoresis, the V9G – chaeDITS-R fragment was used instead. As the amount of target DNA per sample was expected to be very low, some of the PCR products had to be reamplified using a semi-nested PCR approach to obtain the DNA yield

necessary for sequencing, using the internal forward primer ITS5 (White et al., 1990) and one of the two specific reverse primers. PCR reactions were performed using 1.1× ReddyMix PCR Master Mix (ABgene, UK). Primer sequences and detailed PCR protocols are listed in Table S2 (supplementary information).

Ant workers: For ant colonies for which we could not investigate their queens, we determined the ant species by assigning *Azteca* workers to morphologically identified *Azteca* queens by comparing the single-copy nuclear gene Long-Wavelength Rhodopsin (LWRh) according to Pringle et al. (2012). To identify the *Azteca* workers collected in our study primers LR143F and LR639ER were used (Ward and Downie, 2005). The PCR protocol (Supplementary information) was adapted from Ward and Downie (2005).

Sequencing: PCR products were checked with gel electrophoresis and purified using an enzymatic PCR cleanup with FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Fermentas, St. Leon-Rot, Germany) (Werle et al., 1994) as described in Voglmayr and Jaklitsch (2008). DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington) and the PCR primers; in addition two primers were used to complete the partial SSU-ITS-partial LSU fragment of the fungal pure cultures, ITS4 (White et al., 1990) and LR3 (Vilgalys and Hester, 1990). Sequencing was performed on an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems).

Identification of mixed sequences on environmental fungal patches: If only one chaetothyrialean symbiont fungus occurred in an environmental sample we got a clear chromatogram from Sanger sequencing. If more than one chaetothyrialean symbiont fungus co-occurred, sequencing chromatograms had ambiguous nucleotide positions. Those strains had to be analyzed for their co-occurring genotypes, which was done by a sequence comparison approach. First we compared the ambiguous alignment positions with the sequences of known genotypes. If one mixed sequence had e.g. 15 wobble positions (K, M, R, S, W, Y) and two single sequences had exactly the one or the other base at these positions, i.e. the consensus sequence of these two single sequences had exactly the same wobbles, we identified these two sequences to be present in the sample.

To confirm the applicability of our approach and to analyze difficult mixed sequences containing at least three genotypes, and/or indels or deletions, we designed specific forward primers as close as possible to the 5' end of the ITS1 to specifically sequence each allele. In these cases two alternative primers were designed ending at the target wobble position with one or the other nucleotide. Using the new forward primers, two PCRs were repeated per sample. If the annealing temperature was ideal for specific primer bindings, the two genotype sequences present in the sample could be revealed by sequencing the products of the two specific PCR reactions. The alternative primers designed for this approach were:

chaeITS16G (5' GGATCATTATCGAGTTAGGGTCTTG 3') and chaeITS16T (5' GGATCATTATCGAGTTAGGGTCTTT 3'); and chaeITS82A (5' GGCGAGCCTGTTTCTAGTAAA 3') and chaeITS82G (5' GGCGAGCCTGTTTCTAGTAAG 3').

Phylogenetic analyses of fungal symbionts

NCBI blast searches at GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) of sequences of pure cultures consistently revealed members of the “domatia symbiont clade” of Chaetothyriales (Voglmayr et al., 2011) as closest matches. For phylogenetic analyses, a matrix containing representative sequences of each OTU of the current study and of the “domatia symbiont clade” of Voglmayr et al. (2011) was produced. *Cladophialophora scillae* [EU035412] and *Cladophialophora hostae* [EU035407] were chosen as outgroups. The sequences were aligned using Muscle version 3.8.31 (Edgar, 2004) and checked using BioEdit version 7.2.5. (Hall, 2013).

For phylogenetic reconstruction, Bayesian analyses (BA) using MrBayes version 3.2.1 (Huelsenbeck and Ronquist, 2001), Maximum parsimony (MP) bootstrap analyses using PAUP* version 4.0 b10 (Swofford, 2003) and Maximum likelihood (ML) analyses using RAxML (Stamatakis, 2006) were conducted according to Voglmayr et al. (2011) and Nepel et al. (2014). Trees were rooted with the above mentioned *Cladophialophora* species as a monophyletic outgroup.

Molecular identification of *Azteca* ants

Unknown *Azteca* colonies were identified by matching sequences of the nuclear long wavelength rhodopsin (LWRh) (Derocles et al., 2011). Sequencing of the mitochondrial cytochrome c oxydase subunit I (COI) resulted in ambiguous chromatograms of overlain sequences in other species of the genus *Azteca* (pers. experience) and were therefore not considered. Known LWRh sequences of *Azteca* species were taken from GenBank and aligned with our obtained sequences of morphologically identified *Azteca* queens and unknown worker specimens (Fig. S1, supplementary information).

Fungal diversity at the genotype and species (OTU) levels

Genotypes were clustered in operational taxonomic units (OTUs) at 99% similarity cut-off, based on the intraspecific variation of fungal ITS belonging to Eurotiomycetes (Schoch et al., 2012).

Statistics

As a measure of similarity of fungal symbionts among the ant colonies we applied an Analysis of Similarities (ANOSIM) based on a Sørensen matrix for fungal genotypes and OTUs. To evaluate the effect of ant species, plant species or collection site on the composition of fungal symbionts, we conducted a distance-based redundancy analysis on OTU level (Legendre and Anderson, 1999). All multivariate analyses were conducted with PRIMER 7 (Clarke et al., 2015) and the PERMANOVA add-on (Anderson et al., 2008).

Results

The complete list of GenBank accession numbers for the obtained fungal genotypes and all ant sequences can be found in Table S1.

Molecular phylogenetic analysis of the *Cecropia/Azteca* domatia fungi

Of the 563 characters included in the analyses, 121 were parsimony informative. The phylogram of the best ML tree (lnL = -2233.5928) revealed by RAxML is shown in Fig. 2. The three Bayesian runs revealed almost identical posterior probabilities fully compatible with the ML tree. All *Chaetothyriales* genotypes sequenced from the domatia of *Cecropia* inhabiting *Azteca* ants were contained within the domatia symbiont clade described in Voglmayr et al. (2011). The *Azteca* symbionts do not form a monophylum but are scattered within the domatia symbiont clade. All OTUs revealed in the present study seem to be undescribed species.

Fungal diversity

Genotype and species (OTU) level. We isolated Chaetothyriales pure cultures from three different living stems containing *Azteca* ant colonies (one from *A. alfari*, one from *A. constructor*, one from *A. xanthochroa*). Microscopic investigation of the fungal patches showed the dominance of chaetothyrialean hyphae. The molecular analysis of the Chaetothyriales from all samples (pure cultures and environmental ones) resulted in 12 genotypes which differed from each other by 1-25 mutations. They clustered in 6 OTUs (Fig. 2, Table 1). Within OTU1 to OTU4 genotypes differ by 1-3 mutations. OTU5 and OTU6 are single genotypes, each from a single ant colony (Table 1). From 29 *Azteca* colonies in total (11 *A. alfari*, 3 *A. coeruleipennis*, 10 *A. constructor*, 5 *A. xanthochroa*) more than half (65%) were associated with a single fungal OTU, while the remaining samples had more than one OTU (up to 3 genotypes and 3 OTUs; Tables 1, 2A).

We did not find a single dominant omnipresent OTU, but OTU2 and OTU3 were more frequent than others (Fig. 3, Table 2A).

In carton building colonies (*A. constructor* and *A. xanthochroa*), fungal hyphae and conidiophores of the same OTU were found in both fungal patches and in the carton walls (Fig. 1F-H).

Associations among plant species, ant species, region and genotype/OTU

Plant species. We did not find a significant association between *Cecropia* species and fungal genotypes or OTUs (ANOSIM: Global R = 0.024, p = 0.296 for OTU). Only weak trends could be seen: from *C. cf. insignis* and *C. cf. peltata* we isolated OTU2 more frequently than the other OTUs, and *C. obtusifolia* more often had patches with OTU3 (Table 2B). Except for the *C. obtusa* and *C. palmata* samples from French Guiana, in the three other *Cecropia* species 2 – 5 fungal genotypes – OTUs were detected (Table 2B).

Ant species. Significant associations of ant species and fungi occurred at genotype and OTU levels (ANOSIM: Global R = 0.553, $p < 0.001$ on OTU level; Fig. 4, Table 3). The clearest differences were observed between *A. alfari* and the other three *Azteca* species ($p < 0.05$; Table 3). In 10 of 11 *A. alfari* colonies investigated, OTU2 was the sole chaetothyrialean fungus. In general, 64% of all OTU2 samples were from *A. alfari* colonies. OTU2 was also found in the three *A. coeruleipennis* colonies, but in contrast to *A. alfari* it was not the only fungus in their patches. OTU1 was exclusively found in *A. coeruleipennis*, *constructor* and *xanthochroa* colonies and was not associated with *A. alfari* colonies. OTU3 was found in most of the 10 *A. constructor* colonies, in 4 *A. constructor* colonies it was the only fungus found. 58% of all OTU3 samples were from *A. constructor* colonies (Table 1, 2). OTU4 was restricted to *A. constructor*, OTU5 and OTU6 to *A. coeruleipennis*. OTUs were either alone (OTU2 in 63% of the *A. alfari* samples, OTU3 in 40% of the *A. constructor* colonies) or together with other OTUs.

Region. Collection site and fungal diversity were not significantly associated (ANOSIM: Global R = 0.078, $p = 0.085$ on OTU level). But comparing the regions in pairwise tests gave a significant difference between the lowland region of La Gamba and the mountain region of Monteverde ($p < 0.05$, Table 3). OTU1 was exclusively found at higher elevations, in Monteverde. OTUs 2, 3 and 4 occurred in *Azteca* colonies from all collection sites (La Gamba, French Guiana and Monteverde) (Table 3). Only two genotypes (chaeD-CR-4 and chaeD-CR-8) could be found in both Monteverde and La Gamba (Table 3).

A distance based linear model (dbRDA) based on the Sørensen similarity matrix supported the significant influence of ant species ($p = 0.001$, Pseudo-F = 9.79) and region ($p = 0.037$, Pseudo-F = 2.55) on the identity of associated fungal OTUs (Fig. 4).

Morphology of the fungal patches and hyphae

The morphology of the hyphae found in the *Cecropia/Azteca* association was similar to the fungal symbionts observed in other plant domatia, as illustrated in Voglmayr et al. (2011), and in general typical for Chaetothyriales (Fig. 1). On closer examination some differences were observed between different samples. In *Cecropia* occupied by *A. alfari* or *A. coeruleipennis*, the hyphae and conidiophores of the domatia symbiont fungi were concentrated in more or less confined patches (Fig. 1 A, B). *Azteca constructor* and *A. xanthochroa* formed central carton-like nests inside the hollow stem (Fig. 1F). These nests were built by the ants with the spongy tissue from the inner walls of the domatia (Fig. 1 F, G). The carton was usually colonised by the symbiont fungi and spiked with long slender conidiophores, consisting of elongate cells (Fig. 1H). The substrate hyphae were often subhyaline with a conspicuous, thick gelatinous sheath (Fig. 1I) (belonging to OTU3). The conidiophores of fungal symbionts occupied by *A. alfari* (OTU2) were blunt and short, consisting of isodiametric cells (Fig. 1E) and the substrate hyphae were brown and moniliform (Fig. 1 C, D).

Discussion

For the first time the occurrence of domatia fungi was investigated in the *Azteca* – *Cecropia* ant-plant association. We found chaetothyrialean fungi in the hollow stems of all *Cecropia* plants inhabited by symbiotic *Azteca* ants (*A. alfari*, *A. coeruleipennis*, *A. constructor*, *A. xanthochroa*). Though the *Azteca* – *Cecropia* association is one of the best studied ant-plant interactions and has been investigated for more than 100 yr (Belt, 1874; Müller, 1880; Bailey, 1922; Janzen, 1969; Davidson and Fisher, 1991; Yu and Davidson, 1997; Agrawal, 1998; Valverde and Hanson, 2011; Bischof et al., 2013), the frequent occurrence of small chaetothyrialean fungal patches had up to now gone unnoticed. Chaetothyriales have been detected in other ant-plant associations (Voglmayr et al., 2011) and

in fungus gardens of leaf cutter ants (Duarte et al., 2014; Attili-Angelis et al., 2014). Our findings support the ubiquitous occurrence of this group of fungi in ant-plant associations in the tropics.

Our culture-independent analysis revealed that six fungal OTUs occur in colonies of the four *Azteca* species investigated. They clustered in the clade formed by Chaetothyriales living obligatorily in ant nests (domatia symbiont clade) established by Voglmayr et al. (2011). In the phylogram, the *Azteca* cultivated strains did not form a monophyletic group but instead are scattered across those cultivated by ants of non-related ant-plant associations from Africa (*Tetraponera*), Asia (*Cladomyrma*, *Crematogaster*) and South America (*Pseudomyrmex*). However, the whole “domatia symbiont clade” is composed of closely related species.

What is the evolutionary history of the association between chaetothyrialean fungi and plant-ants? Though ants and plants have coexisted for at least 120 Myr (Brady et al., 2006; Bell et al., 2010; Moreau and Bell, 2013) a recent dated phylogeny of the crown groups of 46 myrmecophyte lineages indicates that they arose in the mid-Miocene, are not much older than 16 Myr, and that ant-plant symbioses have evolved at least 150 times independently (Chomicki and Renner, 2015). Ant-plant symbioses have evolved recently, at a time when Africa, America and South-East Asia were already geographically isolated from each other. So the question arises how a set of closely related fungal species have become obligatory symbionts of ants that are geographically and phylogenetically so distant. We propose two hypotheses, although not exclusive: (1) Plant-ant-Chaetothyriales symbiosis evolved once and spread through host switching, in concert with adaptive radiation of the fungal symbiont. This would imply that the fungi show efficient long-distance dispersal. (2) Closely related chaetothyrialean fungal symbionts have been acquired independently in different ant-plant associations on different continents, recruited or evolved from a pool of free-living species in the environment. If such a pool exists it remains to be discovered. All ant domatia symbiont fungi are closely related to each other, which suggests that if hypothesis 2 is correct, the

species in the free-living pool must be pre-adapted for living in association with ants. All representatives of the domatia symbiont clade represent undescribed species from an undescribed chaetothyrialean genus. However, formal description of the species from pure culture is beyond the scope of this study and a task for the future.

Relation between fungal OTU and plant and ant species

It is known that *Azteca* species do not prefer particular *Cecropia* species (Longino, 1991a; Yu and Davidson, 1997). Likewise the fungal OTUs we studied were not associated with particular plant species. In contrast, the fungi were associated with particular ant species. The associations were not exclusive, but rather a tendency for OTUs to form non-random distributions among *Azteca* species. *Azteca alfari* was preferred by one OTU, and was more often found with that single OTU. In contrast, the other three species were more often found with other OTUs and often with more than one OTU in a colony. In other ant-plant-fungus systems investigated so far (*Petalomyrmex phylax/Leonardoxa africana* and *Tetraponera aethiops/Barteria fistulosa*), two fungal strains were regularly detected (Blatrix et al., 2013b).

More than one OTU being present in a mature ant colony may indicate former multi-queen colonization, which is relatively common in *Cecropia* ants (Longino, 1989, 1991a). We observed up to seven *A. xanthochroa* queens in a single domatium and rather often *Azteca* queens from different species in the same *Cecropia* domatium. Pleometrosis (multiqueen founding) was common in the Monteverde highlands but rare in the lowlands of La Gamba. This correlates with Monteverde colonies tending to have multiple OTUs and La Gamba colonies tending to have fewer or, most often, only single OTUs. It is unknown whether foundress queens carry hyphal parts from their natal colony when dispersing. The pattern of OTU distribution along the *Azteca* species suggests that a transmission from mother to daughter colonies is more plausible than a random contamination of the queens from the environment. The fact that *A. alfari* colonies from three different geographic regions cultivate

fungi of OTU2 and *A. constructor* predominantly fungi of OTU3 indicate specificity and vertical transmission between colonies, at least for these two species.

The reason for the difference in fungus diversity between *A. alfari* and the other three species is not clear yet. It could be due to differences in the ants' weeding behaviour which may lead to a stronger selection in *A. alfari* tended fungal patches. The four *Cecropia* inhabiting *Azteca* species are not monophyletic, and *A. alfari* in particular is phylogenetically and behaviorally distant from the other three (Ayala et al., 1996). *Azteca alfari* is not as aggressive as the other three *Cecropia* symbionts and does not defend the host tree as vigorously (Longino, 1991). Due to their lower aggressiveness their hunting success may be lower than in the other species. If carbohydrate rich pearl and Müllerian bodies are scarce they may feed on fungi as described for *Petalomyrmex* living in association with the West African tree *Leonardoxa* (Blatrix et al., 2012). Thus *A. alfari* may be more dependent on fungi than more aggressive *Azteca* species that have access to more prey. Thus, efficient weeding and caretaking of the symbiont fungus would be more important for their colony survival.

Relation between fungal OTU and geographical region

A clear differentiation exists between the fungal OTUs found at the regions of La Gamba and Monteverde. Several OTUs were only found in ant colonies from higher elevation habitats, in Monteverde. These strains may prefer lower temperatures than the ones found in hot lowland forest habitats. The other OTUs seem to have a broader ecological amplitude to climatic conditions, being found in both lowland and upland habitats.

Role of the fungi

Bailey (1922: 388) noted that in *Cecropia angulata* “ants have to contend with luxuriant growths of fungi which obstruct the cavities and interfere with the brood unless they are held in check. The ants trim away the hyphae and cut back the substratum upon which these organisms tend to grow.” We cannot confirm this observation for the colonies investigated in this study. The fungus we found was in most cases in clearly encircled patches (Fig. 1B, F) or

conidiophores were visible on “carton” structures and in older domatia on the domatia wall (Fig. 1E, G). In contrast to the luxuriant growth described by Bailey (1922), the fungi of the associations investigated here were inconspicuous and easy to overlook. Also we cannot confirm that the ants cut back the substratum to prevent fungal growth. Von Ihering (1907) suggested that ants feed upon the spongy parenchyma from immature internodes and interpreted the cutting away of the softer portions of the pith down to a hard, smooth peripheral layer of medullary tissue as a “process of house cleaning”. In contrast to Bailey (1922) and Von Ihering (1907) we instead observed that the ants scrape the parenchyma from the domatia walls and deposit it on the fungal patches. Since the ants also deposit dead nestmates and other organic waste particles on those patches we assume that parenchyma and organic waste keep the fungus growing.

It has recently been shown in the *Leonardoxa/Petalomyrmex* ant-plant association that ants protect their fungal symbiont, nourish it with organic matter (Defosse et al., 2011) and feed its hyphae to their larvae (Blatrix et al., 2012). We, therefore, interpret the parenchyma scraping behaviour as providing a carbon source, and supplementing with dead nestmates and organic waste provides nitrogen. This enhances fungal growth and ensures a nutrient supply to the larvae.

However, a closer look at the *Cecropia-Azteca* mutualism suggests that the reason for fungus cultivation may be different from the *Leonardoxa/Petalomyrmex* system. Myrmecophytic *Cecropia* plants produce Müllerian bodies, glycogen rich food bodies (Müller, 1876, 1880; Bischof et al., 2013). Such food bodies are usually fed to larvae in other ant-plant mutualisms (Fischer et al., 2002). This poses the question of whether fungal hyphae may be used as larval food only under Müllerian body limitation.

Prospect

The group of *Cecropia-Azteca* ant-plant symbioses is highly promising for elucidating the role of fungi in ant-plant symbioses. Many of the *Cecropia-Azteca* symbioses have a large

geographic range and are common along roadsides and in urban areas, and easy access to large colonies is possible. The next steps in the study of interactions between *Azteca* ants, *Cecropia* plants and their symbiotic fungi should be to (1) investigate the process of inoculation by the queen, and (2) disentangle the nutrient flow between ants, fungi and plants. This will elucidate whether fungi play a role in the nutrition of the ant colony, like in other systems, despite the nutrient rich food bodies offered by the plant. It will also help explain distributional patterns of chaetothyrialean fungi in ant-plant associations.

Acknowledgments

We cordially thank the staff at the Estacion Tropical La Gamba and Marvin Hidalgo from the Biological Station Monteverde for the excellent working conditions and their help. The Faculty of Life Sciences from the University of Vienna is thanked for support with the travel costs of M.N. and V.E.M. Data acquisition involved the “Service de microscopie électronique et analytique de l’Université de Montpellier”. Javier Guevara from Sistema Nacional de Áreas de Conservación (SINAC) gratefully did an efficient work with the permits. All collections and exportations were under permission from SINAC – Sistema Nacional de Areas de Conservación de Costa Rica of the Ministry of Environment and Energy (MINAE) to M.N. and V.E.M. (No. 183-2010-SINAC, 051-2012-SINAC, 142-2013-SINAC). JTL's contribution was supported by National Science Foundation grant DEB-1354739 (Project ADMAC).

References

- Agrawal, A.A., 1998. Leaf damage and associated cues induced aggressive ant recruitment in a neotropical ant-plant. *Ecology* 79, 2100–2112.
- Anderson, M., Gorley, R.N., Clarke, R.K. 2008. Permanova+ for Primer: Guide to software and statistical methods. Primer-E, Plymouth, UK.

- Attili-Angelis, D., A. P. M. Duarte, F. C. Pagnocca, N. S. Nagamoto, M. Vries, J. B. Stielow, Hoog, G. S. 2014. Novel *Phialophora* species from leaf-cutting ants (tribe Attini). *Fungal Div.* 65, 65–75.
- Ayala, F.J., Wetterer, J.K., Longino, J.T., Hartl, D.L., 1996. Molecular phylogeny of *Azteca* ants (Hymenoptera: Formicidae) and the colonization of *Cecropia* trees. *Mol. Phyl. Evol.* 5, 423–428.
- Bailey, I.W., 1920. Some relations between ants and fungi. *Ecology* 1, 174–189.
- Bailey, I.W., 1922. Notes on Neotropical ant-plants. I. *Cecropia angulata*, sp. nov. *Bot. Gaz.* 74, 369–391.
- Bell, C.D., Soltis, D.E., Soltis, P.S., 2010. The age and diversification of the angiosperms revisited. *Am. J. Bot.* 97, 1296–1303.
- Belt, T., 1874. *The Naturalist in Nicaragua*. Reprint 2015, Andesite Press, UK.
- Bequaert, J., 1922. Ants in their diverse relations to the plant world. *Bull. Am. Mus. Nat. Hist.* 45, 333–583.
- Berg, C., Franko Rosseli, P., 2005. *Cecropia*. New York Botanical Garden Press, New York.
- Bischof, S., Umhang, M., Eicke, S., Streb, S., Qi, W., Zeeman, S.C., 2013. *Cecropia peltata* accumulates starch or soluble glycogen by differentially regulating starch biosynthetic genes. *Plant Cell* 25, 1400–1415.
- Blatrix, R., Djéto-Lordon, C., Mondolot, L., La Fisca, P., Voglmayr, H., McKey, D., 2012. Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant–plant interactions. *Proc. R. Soc. London Biol. Sci.* 279, 3940–3947.
- Brady, S.G., Schultz, T.R., et al. 2006. Evaluating alternative hypotheses for the early evolution and diversification of ants. *Proc. Natl. Acad. Sci. U.S.A.* 103, 18172–18177.
- Caldera, E.J., Poulsen, M., Suen, G., Currie, C.R., 2009. Insect symbioses: a case study of past, present, and future fungus-growing ant research. *Environ. Entomol.* 38, 78–92.

- Chomicki, G., Renner, S.S., 2015. Phylogenetics and molecular clocks reveal the repeated evolution of ant-plants after the late Miocene in Africa and the early Miocene in Australasia and the Neotropics. *New Phytol.* 207, 411–424.
- Chomicki, G., Ward, P.S. & Renner, S.S., 2015. Macroevolutionary assembly of ant/plant symbioses: *Pseudomyrmex* ants and their ant-housing plants in the Neotropics. *Proc. R. Soc. B.* 282, 20152200.
- Clarke, K.R., Gorley, R.N. 2015. Primer 7. Primer-E, Plymouth, UK.
- Danovaro, R., Gambi, C., Dell'Anno, A., Corinaidesi, C., Fraschetti, S., Vanreusel, A., Vincx, M., Gooday, A.J., 2008. Exponential decline of deep-sea ecosystem functioning linked to benthic biodiversity loss. *Current Biol.* 18, 1–8.
- Davidson, D.W., Fisher, B.F., 1991. Symbiosis of ants with *Cecropia* as a function of light regime, in: Huxley CR, Cutler DF (Eds.), *Ant–Plant Interactions*. Oxford University Press, Oxford, UK, pp. 289–309.
- Davidson, D.W., McKey, D. 1993. The evolutionary ecology of symbiotic ant-plant relationships. *J. Hymenoptera Res.* 2, 13–83.
- Defosse, E., Djieto-Lordon, C., McKey, D., Selosse, M.A., Blatrix, R.. 2011. Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. *Proc. R. Soc. London Biol. Sci.* 278, 1419–1426.
- Defosse, E., Selosse, M.A., Dubois, M.P., Mondolot, L., Faccio, A., Djieto-Lordon, C., McKey, D., Blatrix, R., 2009. Ant-plants and fungi: a new threeway symbiosis. *New Phytol.* 182, 942–949.
- Dejean, A., Solano, P.J., Ayroles, J., Corbara, B., Orivel, J., 2005. Arboreal ants build traps to capture prey. *Nature* 434, 973.
- Docters van Leeuwen, W., 1929. Einige Beobachtungen ueber das Zusammenleben von *Camponotus quadriceps* F. Smith mit dem Ameisenbaum *Endospermum formicarum* Becc. aus Neu-Guinea. *Treubia* 10, 431–437.

- Duarte, A. P. M., D. Attili-Angelis, N. C. Baron, L. C. Forti, Pagnocca, F. C. 2014. Leaf-cutting ants: an unexpected microenvironment holding human opportunistic black fungi. *Antonie van Leeuwenhoek* 106, 465-473.
- Hall, T. 2013. BioEdit v7.2.5 <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>.
- Huxley, C.R., 1978. Ant-plants *Myrmecodia* and *Hydnophytum* (Rubiaceae), and the relationships between their morphology, ant occupants, physiology and ecology. *New Phytol.* 80, 231–268.
- Ihering, H. von, 1907. Die Cecropien und ihre Schutzameisen. *Bot. Jahrb. Syst. Pflanzengesch. Pflanzengeogr.* 39, 666–714.
- Janzen, D.H., 1972. Protection of *Barteria* (Passifloraceae) by *Pachysima* ants (*Pseudomyrmecinae*) in a Nigerian rain-forest. *Ecology* 53, 885–892.
- Janzen, D.H., 1969. The ant *Azteca* as an allelopathic agent of *Cecropia*. *Ecology* 50, 147–153.
- Kiers, E.T., Palmer, T.M., Ives, A.R., Bruno, J.F., Bronstein, J.L., 2010. Mutualisms in a changing world: an evolutionary perspective. *Ecol. Letts.* 13, 1459–1474.
- Lauth, J., Ruiz-Gonzalez, M.X., Orivel, J., 2011. New findings in insect fungiculture. Have ants developed non-food, agricultural products? *Communicative Integrative Biol.* 4, 728–730.
- Legendre, P., and Anderson, M. J. 1999. Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments. *Ecol. Monogr.* 69, 1-24.
- Leroy, C., Sejalón-Delmas, N., Jauneau, A., Ruiz-Gonzalez, M.X., Gryta, H., Jargeat, P., Corbara, B., Dejean, A., Orivel, J., 2011. Trophic mediation by a fungus in an ant-plant mutualism. *J. Ecol.* 99, 583–590.
- Letourneau, D.K., 1998. Ants, stem-borers, and fungal pathogens: Experimental tests of a fitness advantage in *Piper* ant-plants. *Ecology* 79, 593–603.

- Longino, J.T., 1991. Taxonomy of the *Cecropia*-inhabiting *Azteca* ants. *J. Nat. Hist.* 25, 1571–1602.
- Mayer, V.E., Voglmayr, H., 2009. Mycelial carton galleries of *Azteca brevis* (Formicidae) as a multi-species network. *Proc. R. Soc. London Biol. Sci.* 276, 3265–3273.
- Miehe, H., 1911. Untersuchungen über die javanische *Myrmecodia*. *Abh. Math.-Phys. Kl. Königl.-Sächs. Ges. Wiss.* 32, 312–361.
- Müller, F., 1876. Über das Haarkissen am Blattstiel der Imbauba (*Cecropia*), das Gemüsebeet der Imbauba-Ameise. *Jenaische Z. Naturwiss.* 10, 281–286.
- Müller, F., 1880. Die *Imbauba* und ihre Beschützer. *Kosmos* 8, 109–116.
- Moreau, C.S., Bell, C.D., 2013. Testing the museum versus cradle biological diversity hypothesis: Phylogeny, diversification, and ancestral biogeographic range evolution of the ants. *Evolution* 67, 2240–2257.
- Nepel, M., Voglmayr, H., Schönenberger, J., Mayer, V., 2014. High diversity and low specificity of chaetothyrialean fungi in carton galleries in a Neotropical ant–plant association. *PLoS One* 9 (11), e112756.
- Pringle, E. G., Ramírez, S. R., Bonebrake, T. C., Gordon, D. M., Dirzo, R. 2012. Diversification and phylogeographic structure in widespread *Azteca* plant-ants from the northern Neotropics. *Mol. Ecol.* 21:3576–3592.
- Rickson, F.R., 1971. Glycogen plastids in Müllerian body cells of *Cecropia peltata* - Higher Green Plant. *Science* 173, 344–347.
- Rico-Gray, V., Oliveira, P.S., 2007. The Ecology and Evolution of Ant-Plant Interactions. University of Chicago Press, Chicago.
- Rosumek, F.B., Silveira, F.A.O., Neves, F.D., Barbosa, N.P.D., Diniz, L., Oki, Y., Pezzini, F., Fernandes, G.W., Cornelissen, T., 2009. Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. *Oecologia* 160, 537–549.

- Ruiz-González, M.X., Male, P.J.G., Leroy, C., Dejean, A., Gryta, H., Jargeat, P., Quilichini, A., Orivel, J., 2011. Specific, non-nutritional association between an ascomycete fungus and *Allomerus* plant-ants. *Biol. Lett.* 7, 475–479.
- Schlick-Steiner, B.C., Steiner, F.M., Konrad, H., Seifert, B., Christian, E., Moder, K., Stauffer, C., Crozier, R.H., 2008. Specificity and transmission mosaic of ant nest-wall fungi. *Proc. Natl. Acad. Sci. U.S.A.* 105, 940–943.
- Schremmer, V.F., 1984. Untersuchungen und Beobachtungen zur Ökoethologie der Pflanzenameise *Pseudomyrmex triplarinus*, welche die Ameisenbäume der Gattung *Triplaris* bewohnt. *Zool. Jahrb., Abt. Syst. Ökol. Geogr. Tiere* 111, 385–410.
- Suarez, A.V., De Moraes, C.M., Ippolito, A., 1998. Defense of *Acacia collinsii* by an obligate and nonobligate ant species: the significance of encroaching vegetation. *Biotropica* 30, 480–482.
- Treiber, E. L., A. L. Gaglioti, S. Romaniuc-Neto, S. Madriñán, Weiblen, G. D., 2016. Phylogeny of the Cecropieae (Urticaceae) and the Evolution of an Ant-Plant Mutualism. *Syst. Bot.* 41, 56–66.
- Valverde, J.P., Hanson, P., 2011. Parenchyma: a neglected plant tissue in the *Cecropia*/ant mutualism. *Symbiosis* 55, 47–51.
- Vazquez, D.P., Bluethgen, N., Cagnolo, L., Chacoff, N.P., 2009. Uniting pattern and process in plant-animal mutualistic networks: a review. *Ann. Bot.* 103, 1445–1457.
- Voglmayr, H., Jaklitsch, W., 2008. *Prosthecium* species with *Stegonsporium* anamorphs on *Acer*. *Mycol. Res.* 112, 885–905.
- Voglmayr, H., Mayer, V., Maschwitz, U., Moog, J., Djieto-Lordon, C., Blatrix, R., 2011. The diversity of ant-associated black yeasts: Insights into a newly discovered world of symbiotic interactions. *Fungal Biol.* 115, 1077–1091.

- Ward, P. S., Downie, D. A. 2005. The ant subfamily Pseudomyrmecinae (Hymenoptera : Formicidae): phylogeny and evolution of big-eyed arboreal ants. *Syst. Entomol.* 30, 310-335.
- Yu, D., Davidson, D.W., 1997. Experimental studies of species-specificity in *Cecropia*-ant relationships. *Ecol. Monogr.* 67, 273–294.

Figure legends:

Fig. 1 – Chaetothyrialean fungi in *Cecropia* stems serving as domatia for *Azteca* ants. (A) Young longitudinally opened stem of *C. insignis* with *A. xanthochroa* (queen and young workers) showing brownish patches and refuse piles colonized by domatia fungi (arrows); (B) Domatium of *C. palmata* inhabited by *A. alfari* showing fungal patch containing debris of ants; (C) Transmission electron microscopy of the interface between fungal patch and inner wall of a domatium of *C. palmata* occupied by *A. alfari*. HC host cells, h hyphal cells of domatia symbiont fungus; note that hyphae do not penetrate the host tissue; (D), (E) Light microscopy of domatia fungi from *C. insignis* inhabited by *A. alfari* showing substrate hyphae (D) and conidiophore (E). (F)-(I) *C. obtusifolia* inhabited by *A. cf constructor*; (F) Cut domatia with “carton” formed from stem parenchyma scraped off the stem wall, serving as brood chambers; (G) Close-up of (F), showing numerous erect, dark brown, spiny conidiophores of the domatia symbiont fungus colonizing the carton structures; (H), (I) Light microscopy of domatia fungi showing brown conidiophore (H) and hyaline substrate hyphae with thick gelatinous sheath (I). Bars: (A), (F) 10 mm, (B) 5 mm, (C)-(E), (H), (I) 10 μ m, (G) 100 μ m.

Fig. 2 – Phylogram of the best maximum likelihood tree (lnL = -2233.5928) revealed by RAxML from an analysis of the ITS rDNA matrix of chaetothyrialean domatia clade fungi, showing the phylogenetic position of the *Azteca* symbiont genotypes within the domatia symbiont clade of Voglmayr et al. (2011). Bold branches indicate ML and MP bootstrap support above 95% and BA PP above 0.99. The tree was rooted by two *Cladophialophora* species as outgroup according to Voglmayr et al. (2011). Accessions in bold were sequenced in the present study. The bar gives the substitution rate per site.

Fig. 3 – Distribution of the four most common OTUs in colonies of *Azteca alfari* (n=11), *A. coeruleipennis* (n=3), *A. constructor* (n=10) and *A. xanthochroa* (n=5).

Fig. 4 – Constrained ordination plot (dbRDA) based on the Sørensen similarity matrix of fungal symbiont genotypes. The significant influence of ant species and region is supported.

Figure 1

Figure 2

Figure 3

Figure 4

Table 1

Table 1 – Genotypes and OTUs in patch and carton samples of *Cecropia* trees inhabited with *Azteca* ants from Monteverde, Costa Rica (CR13), La Gamba, Costa Rica (CR11 and CR12) and Kourou, French Guiana (FG). Names above the OTU number are names of the genotypes (sequence identifiers).

Sample no	<i>Cecropia</i> species	<i>Azteca</i> species	OTU1	OTU2	OTU3	OTU4	OTU5	OTU6
CR11Cec4Jul	cf. <i>insignis</i>	<i>alfari</i>		X				
CR12Cec2	cf. <i>insignis</i>	<i>alfari</i>		X				
CR12Cec3	cf. <i>insignis</i>	<i>alfari</i>		X	X			
CR12 Cec4	cf. <i>insignis</i>	<i>alfari</i>		X				
CR12Cec11	cf. <i>insignis</i>	<i>alfari</i>		X				
CR12Cec18	cf. <i>obtusifolia</i>	<i>alfari</i>		X				
CR12Cec23	cf. <i>obtusifolia</i>	<i>alfari</i>		X				
CR13 Cec1	cf. <i>peltata</i>	<i>alfari</i>		X				
CR13Cec10	cf. <i>peltata</i>	<i>alfari</i>		X				
FGCec7	<i>C. obtusa</i>	<i>alfari</i>			X			
FGCec12	<i>C. palmata</i>	<i>alfari</i>			X			
CR13 Cec14	cf. <i>insignis</i>	<i>coeruleipennis</i>		X	X			X
CR13Cec20	cf. <i>insignis</i>	<i>coeruleipennis</i>	X	X				
CR13Cec25	cf. <i>peltata</i>	<i>coeruleipennis</i>		X			X	
CR12Cec14	cf. <i>insignis</i>	<i>constructor</i>			X			
CR11Cec2Feb	cf. <i>obtusifolia</i>	<i>constructor</i>			X			
CR12Cec15	cf. <i>obtusifolia</i>	<i>constructor</i>			X		X	
CR12Cec20	cf. <i>insignis</i>	<i>constructor</i>					X	
CR12 Cec25	cf. <i>obtusifolia</i>	<i>constructor</i>			X			
CR12Cec26	cf. <i>obtusifolia</i>	<i>constructor</i>			X			
CR13Cec3	cf. <i>obtusifolia</i>	<i>constructor</i>	X					
CR13Cec4	cf. <i>obtusifolia</i>	<i>constructor</i>	X	X		X		
CR13Cec6	cf. <i>obtusifolia</i>	<i>constructor</i>	X			X	X	
CR13Cec24	cf. <i>obtusifolia</i>	<i>constructor</i>	X					
CR13Cec9	cf. <i>peltata</i>	<i>xanthochroa</i>	X	X				
CR13Cec11	cf. <i>peltata</i>	<i>xanthochroa</i>			X			
CR13Cec12	cf. <i>insignis</i>	<i>xanthochroa</i>		X	X			
CR13Cec18	cf. <i>obtusifolia</i>	<i>xanthochroa</i>	X		X			
CR13Ceci2	cf. <i>insignis</i>	<i>xanthochroa</i>						

Table 2

Table 2a – OTUs found in colonies of *Cecropia* inhabiting *Azteca* species. In more than half of all *Azteca* colonies investigated (n=29) only one OTU was found.

	Colony number	OTU 1	OTU 2	OTU 3	OTU 4	OTU 5	OTU 6	No of colonies with > 1 OTU
Σ ant colonies	29	8	17	12	3	1	1	10
<i>A. alfari</i>	11		11	1				1
<i>A. coeruleipennis</i>	3	1	3	1		1	1	3
<i>A. constructor</i>	10	4	1	7	3			3
<i>A. xanthochroa</i>	5	3	2	3				3

Table 2b – OTU distribution in the five *Cecropia* species.

	Plant number	OTU 1	OTU 2	OTU 3	OTU 4	OTU 5	OTU 6
Σ plant species	29	3	5	3	2	1	1
<i>C. cf. insignis</i>	11	2		4	1	1	
<i>C. palmata</i>	1		1				
<i>C. cf. peltata</i>	5	1		1			1
<i>C. obtusa</i>	1		1				
<i>C. cf. obtusifolia</i>	11	5	3		2		

Table 3

Table 3 – Association between fungal OTUs, *Cecropia* plant species, *Azteca* ant species and collection site assessed by Analysis of Similarities (ANOSIM, 999 permutations) based on a Sørensen similarity matrix. Significant results ($p < 0.05$) printed in bold.

Global test	R	p
Plants	R = 0.024	p = 0.296
Ants	R = 0.553,	p < 0.001
Regions	R = 0.078	p = 0.085
Pairwise tests ant species		
<i>alfari, coeruleipennis</i>	0.788	0.088
<i>alfari, constructor</i>	0.767	0.001
<i>alfari, xanthochroa</i>	0.792	0.011
<i>coeruleipennis, constructor</i>	0.326	0.045
<i>coeruleipennis, xanthochroa</i>	0.103	0.286
<i>constructor, xanthochroa</i>	-0.055	0.626
Pairwise tests region		
La Gamba, Monteverde	0.116	0.043
La Gamba, French Guiana	-0.130	0.924
Monteverde, French Guiana	0.006	0.458