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„Diversity in fungal patches of *Cecropia-Azteca* associations“

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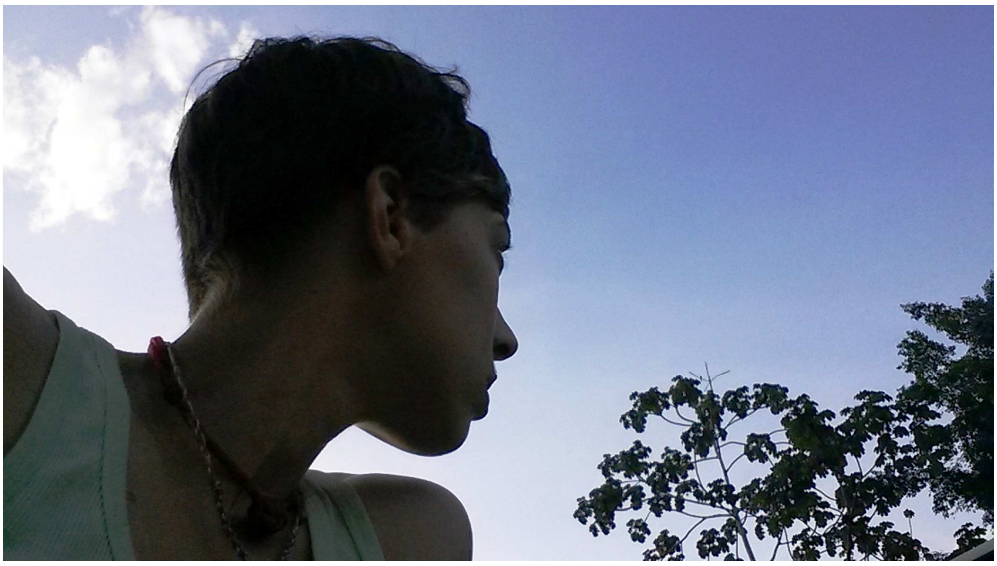

‘If I were a cecropia, I´d want some of these ants living on me.’

- John Kricher in: A Neotropical Companion

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To get through UNI was quite rough,
life was not easy and often tough.
YOU held my back and were always there,
when I was down- I knew you care.
But also in the happy times,
we shared laughter and some funny rhymes.
YOU are my supporting crowd,
to have you in my life just makes me proud!

Vielen herzlichen Dank an meine Oma, Helga, die sich jederzeit um meine seelisch moralische, aber vor allem kulinarische Versorgung gekümmert hat. Letzte dankbare Worte richte ich an Silko, der mich vor allem im letzten Jahr emotional unterstützt, und alle meine Launen mit einem Optimismus sondergleichen ertragen hat.

– THANK YOU, DANKE.

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Abstract

The association of *Cecropia* sp. (Urticaceae) with *Azteca* sp. (Formicidae: Dolichoderinae) has long been a classical example for ant-plant associations. The plants provide ants with glycogen-rich food bodies (Müllerian bodies), pearl bodies on the leaf surface and nesting space in the hollow stem internodes (so-called “domatia”). In return for shelter and food, the *Azteca* ants react with impressive aggression towards herbivores and overgrowing plants. But the *Cecropia-Azteca* association is more than a bipartite interaction. Beside the host-plant and the inhabiting ants, also nematodes, coccids, bacteria and fungi play a major role in the mutualism. The fungi were only recently discovered and assigned to the ecologically rather unusual order of Chaetothyriales (Ascomycetes) which are often found in extreme environmental niches. In arboreal ant-plant associations, chaetothyrialean fungi seem to be ubiquitous, and have a global occurrence. Most fungal strains found so far in ant-plant interactions are new to science, still need to be taxonomically described, and are currently assigned to so-called OTUs (operational taxonomic units = cluster of closely related genotypes). Chaetothyrialean fungi occur inside the domatia as well as outside on ant made nests and “runway galleries”, where the dense hyphal net has a stabilizing function for the nest and gallery walls. On these carton-like walls a high diversity of different chaetothyrialean OTUs is present. In contrast, much fewer OTUs were found in small, fungi containing “patches” inside the domatia of tropical ant-plants (myrmecophytes). Also inside the hollow stem of the Neotropical *Cecropia* sp. trees, fungal patches are found, tended by *Azteca* sp. ants.

In order to disentangle the phylogeny of Chaetothyriales and to learn more about the chaetothyrialean strains living in association with tropical arboreal ants, ongoing studies focus on the diversity of those fungi in ant-nests. In the present study, the diversity of genotypes and OTUs in *Cecropia-Azteca* associations was investigated with molecular methods. Also preferences of different *Azteca* sp., especially *A. constructor* and *A. alfari*, for specific chaetothyrialean fungi were analysed. For phylogenetic assignment, a phylogenetic tree including chaetothyrialean sequences from previous studies was reconstructed. Chaetothyrialean DNA from 37 fungal patch and ‘carton’ samples, from different *Cecropia-Azteca* associations, was gained successfully with analysis of the partial 18S (SSU), the complete ITS1-5.8S-ITS2 unit (ITS) and parts of the 28S (LSU) of the ribosomal DNA. All sequences were assigned to OTUs of the domatia-clade in the phylogenetic analysis, confirming low chaetothyrialean diversity inside *Cecropia* domatia. 70% of fungal sequences from *A. constructor* were assigned to OTU3, 25% to OTU2 and one sequence was found sister to a South-Asian ant-plant-fungi association, which indicates a global distribution of ant specific chaetothyrialean fungi. 53% of *A. alfari*-tended samples were assigned to OTU2, 40% to OTU3 and one sequence was assigned to OTU1. Hence, OTU-ant specificity can be confirmed for *A. constructor* and *A. alfari*. Low chaetothyrialean diversity inside domatia and given OTU-ant specificity lead to the hypothesis of vertical fungi-transmission. Alate queens may carry hyphae parts from their mother colony during the nuptial flight and inoculate their new fungal patches with the hyphae they bring along. A similar behaviour has been shown for young Attini queens.

This study offers new insights into the distribution of chaetothyrialean fungi in fungal patches inside domatia of the Neotropical *Cecropia* sp. with *Azteca* sp. ant hosts. Hence, it adds another piece of the evolutionary and functional puzzle of the Chaetothyriales-ant-plant symbiosis. To answer questions on the evolution of ant-plant-fungus interactions further studies with similar associations should be attempted.

(**Keywords**: *Cecropia*, myrmecophyte, *Azteca*, La Gamba, Chaetothyriales, black yeasts)

Introduction

Currently, the scientific study of ants (myrmecology), including studies on ant-plant interactions, is one of the most exciting disciplines that a student and researcher can attempt to understand. Although mutualistic ant-plant interactions have been known for a long time (e.g. Keller, 1892), they have been met with a steadily rising interest in science since Daniel H. Janzen´s experimental research on *Acacia* plants and *Pseudomyrmex* ants in 1966. Much progress has been made in understanding these eusocial insects and their interactions with the fauna and flora surrounding a colony in recent years (Rico-Gray and Oliveira 2007, Hölldobler and Wilson 2016).

Furthermore, recent investigations revealed that ant-plant interactions are not only bipartite symbiosis between ants and their host plants, but often are multi-partner mutualisms. These mutualisms include not only the plant and the ants, but also coccids, nematodes, bacteria and fungi (Heckroth *et al.* 1998, Voglmayr *et al*. 2011, Seipke *et al.* 2013, Maschwitz *et al.* 2016). Simultaneously with technical progress, the focus of studies with ant-plant interactions shifts from obvious partners, observable with bare eye, to those only visible through "visual enlargers" like microscopes (e.g. Blatrix *et al.* 2009) or "translaters" like molecular studies (e.g. Seipke *et al.* 2013, González-Teuber *et al.* 2014 and others).

Nevertheless, the question whether all microorganisms play a minor or major role in the mutualism, remains only partly answered so far. The study presented here contributes to the knowledge about diversity and specificity of fungi in the *Cecropia-Azteca* associations, one of the most famous and best investigated ant-plant interactions so far. As mentioned by Warburg (1892), ants housing in *Cecropia* stems were noticed first by Marcgravius in 1648. Whereas fungi in *Cecropia-Azteca* mutualisms were only relatively recently discovered and studied, fungi have been a well-known symbiont with the ant tribe Attini for many years. Fungi cultivated on organic leaf material by Attini ants mostly belong to the Leucocoprini (Basidiomycotina: Agaricales: Lepiotaceae). The monocultures of basidiomycetes serve solely as the major food source for Attini ants (Hervey *et al.* 1977, Chapela *et al.* 1994, Mueller *et al.* 1998, 2010).

More recent studies have been looking into ant-plant-fungus interactions of non-attine ants (Nepel *et al.* 2014 and 2016, Kokolo *et al.* 2016, Vasse *et al.* 2017). Whereas Attini ants only occur in the New World (Jolivet 1996), arboreal ants with fungiculture are more widely distributed. Studies with samples from tropical zones from Central and South America, Africa, and Southeast Asia, showed a broad diversity of fungal species, occurring in associations with ants and their host plants (Voglmayr *et al.*, 2011; Vasse *et al.* 2017). Even in the temperate zone, the European ant *Lasius fuliginosus* builds carton structures, in which observed fungal hyphae are apparently cultivated to function as stabilizers of the nest walls and as a food source (Escherich 1906, Maschwitz and Hölldobler 1970, Schlick-Steiner *et al.* 2008).

Fungi in ant-plant associations, other than the Attini-tribe, belong mostly to the order Chaetothyriales (Ascomycetes), also called “black yeasts” (Ascomycota, Euromycetes), and seem to have a global distribution (Voglmayr *et al.* 2011, Nepel *et al.* 2013, Vasse *et al.* 2017). During previous investigations of fungi in carton structures, Vasse *et al.* (2017) described isolated fungal strains of other orders than Chaetothyriales or Capnodiales as most likely to be contaminants in ant-Chaetothyriales interactions (Nepel *et al.* 2016, Vasse *et al.* 2017). Black yeasts and relatives are known to exist in oligotrophic or extreme environments, like on the surface of bare rocks, plant surfaces, indoor surfaces of buildings and substrates contaminated with aromatic hydrocarbons (Prenafeta-Boldú *et al.* 2006, Gueidan *et al.* 2014). Chaetothyriales are also subjects of studies in animal and human medical science, as they are opportunistic animal and human pathogens, causing severe chromoblastomycoses and phaeohyphomycoses (de Hoog 2000). Although, black yeasts can exist in very rough environments, like on bare rocks in the Antarctica (Selbmann *et al.* 2005), they seem to be weak competitors in mild and buffered surroundings (Zhao *et al.* 2010). Because ants produce toxic substances in their glands, every environment occupied by them is rather unfavourable for other organisms (Schlüns and Crozier 2009, Voglmayr *et al.* 2011). Hence, a preadaptation of Chaetothyriales for ant-provided niches can be suggested (Nasciemento *et al.* 2016, Vasse *et al.* 2017).

The mutualism between ants and fungi seems to base on the ant’s ability to shape their microbial environment. At least, this is the case with attine-ants and their agriculturing behaviour, as published by Mueller *et al.* (2005).

Chaetothyrialean fungi are cultivated by ants in their nests. Most tropical ants have their nests inside plants. So called ant-plants provide nesting spaces, called “domatia”, in hollow stems, branches, leaf pouches or other structures of the plant body (Jolivet 1996, Nepel *et al.* 2016). Ant-plant symbioses involving domatia structures are common in tropical regions, but do not occur under other climatic conditions. Some myrmecophytic plants not only offer domatia, but also nutrient rich food sources, as either solid food bodies, or liquid drops from extrafloral nectaries. In return for feeding the resident ants, the plant can absorb nutrients such as nitrogen from deposited ant debris and will be defended against herbivores by the ants (Sagers *et al.* 2000, Fischer *et al.* 2002, Gegenbauer *et al.* 2012). In many of these ant-plant associations, fungi can be found inside the domatia in so called "fungal patches" (Fig. 1B), or in interior, ant-made, cardboard-like structures (Fig. 1A). Ant made exterior structures, interwoven with fungal mycelium, are constructed as so called "runway galleries" by *Allomerus* ants (Ruiz-Gonzalez *et al.* 2011) or tunnel-like constructs by *Azteca brevis* ants on living stems of *Tetrathylacium macrophyllum* (Flacourtiaceae) (Mayer *et al.* 2009, Nepel *et al.* 2014). The tunnels and galleries function as protection and traps, to capture larger pray (Dejean *et al.* 2005, Mayer *et al.* submitted). Inside some domatia, e.g. of *Cecropia* sp. (Urticaceae) trees, *Azteca* sp. (Formicidae: Dolichoderinae) ants also build interior 'carton' structures. *A. constructor* and *A. xanthochroa* ants scrape off masticated stem parenchyma of hollow *Cecropia* internodes and, as described in Nepel *et al.* (2016) to form such carton structures, in which the brood is kept (Fig. 1A).

Chaetothyrialean fungi are also the main component in fungal patches cultivated by *Azteca* sp. ants inside their *Cecropia* sp. host tree (Fig. 2B), the study system investigated here.

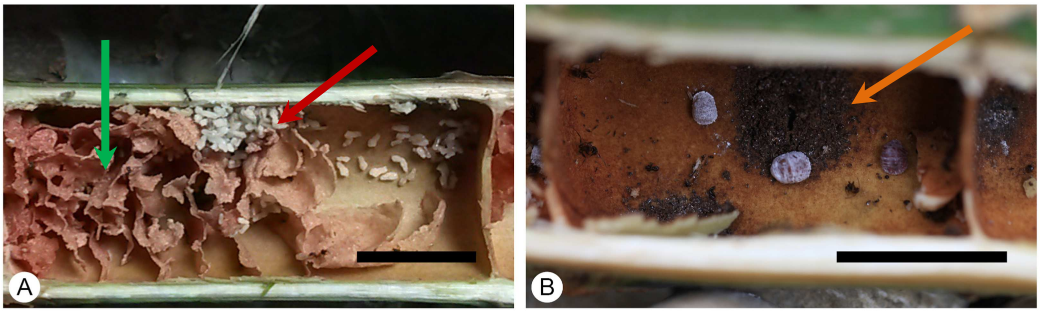

Fig. 1 Chaetothyrialean fungi in *Cecropia* domatia. **(A),** Longitudinally split *Cecropia* stem, showing separated internodes with carton structures (green arrow) and ant larvae (red arrow). **(B)** Inside of an internode with a blackish fungal patch (orange arrow) and white coccids. Bars: (A), 2,5 cm; (B), 1,5 cm. Photos: Mayer V.

The omnipresence of fungi in ant-plant associations is undoubted nowadays (Defossez *et al.* 2009, Mayer and Voglmayr 2009, Voglmayr *et al.* 2011, Vasse *et al.* 2017). The stabilizing function of chaetothyrialean fungi in runway galleries and carton nests was investigated and partly confirmed by multiple studies in the last 10 years (Schlick-Steiner *et al.* 2008, Voglmayr *et al.* 2011, Ruiz-González *et al.* 2011, Nepel *et al.* 2014).

Recently, interest has shifted from more obvious objects, like nests and galleries on the outside of ant-plants, to the ones hidden from the bare eye, because they are located inside the domatia: the fungal patches and interior 'carton' structures created by some *Azteca* species. Studies on trophic relationships between other ant-plant-fungi symbioses show that the ants are the caretakers of the chaetothyrialean fungal patches. While the function of the patches in the *Cecropia-Azteca* mutualisms is not fully understood yet, the role of fungi as food source is now well supported (Defossez *et al.* 2011, Blatrix *et al.* 2012) and thus, the behaviour of *Azteca* ants is comparable with the behaviour of attine ants and their treatment of basidiomycete fungi in their fungal gardens. Studies now focus on phylogenetic relationships of chaetothyrialean fungi in non-attine ant-fungus mutualisms (Mayer *et al.* 2009, Voglmayr *et al.* 2011, Nepel *et al.* 2016, Vasse *et al.* 2017). Fitting the fungi into phylogenetic trees was not the sole target of previous and the present study, but contributes to the insight into evolution of ant-fungi symbiosis, which may date way back into the late Miocene when ant-plant symbioses evolved (Davidson and McKey 1993, Chomicki and Renner 2015; Chomicki *et al.* 2015).

The thesis at hand is an attempt to push forward research on the role of chaetothyrialean fungi in one of the most famous ant-plant interactions: the *Cecropia-Azteca* association, which could serve as model system to detangle the function of the fungal patches also in other ant-plant interactions.

A major goal of this thesis was to investigate the diversity of chaetothyriaelean fungi in fungal patches and on 'carton' structures inside domatia using molecular methods. Additionally, with a few samples, the occurrence of Chaetothyriales on the plants surface of different *Cecropia-Azteca* associations was tested. My hypothesis on low diversity expectations is based on the results of Nepel *et al.* (2016), a study which was based on a smaller sample size.

A second goal was to identify potential preferences of *Azteca constructor* and *A. alfari* for specific, fungal operational taxonomic unit (OTU). Based on the results of Nepel *et al.* (2016), I hypothesize that at least *A. alfari, but perhaps also A. constructor* may have a preference for a specific OTU.

Finally, this thesis aimed at morphologically circumscribing fungal patches with light microscopy and scanning electron microscope and to add to our knowledge on chaetothyrialean fungi in domatia.

Material and methods

Collection area

Investigated material was collected on the pacific side of Central America in the region Golfo Dulce, SW Costa Rica near the tropical research facility “Tropenstation La Gamba” (N08°42’03’’, W083°12’06’’, 70m asl).

The “Tropenstation La Gamba” is situated next to the Parque Nacional Piedras Blancas at the base of a lower mountain ridge. The national park covers 148km² with the Cerro Nicuesa as its highest peak with 579m and is directly connected to the Golfo Dulce. Primary rainforest covers many narrow ridges and steep slopes. Abandoned fields in the lowlands are covered by secondary forest at different developmental stages (Weissenhofer 2005).

The collection area is amid one of the wettest lowland rainforests in Central America (Aschan 1996). In 2007 there were only 80 days without rain recorded at the “Tropenstation La Gamba” and based on meteorological data recorded between 1998 and 2007, the average annual precipitation was 5.836mm per year. The average yearly temperature measured was 28.2 °C with the coolest month being December (average temperature of 27.3 °C) and the warmer months being April and May (average temperature of 29.9 °C). The warm temperature and the high amount of rainfall result in relative high average humidity of 88.3% throughout the year at the research facility and is even higher (97.7%) within the surrounding forest (Aschan 1996, Weissenhofer and Huber 2008).

Samples used for this study were collected in August 2015 and February 2016. All samples were collected either close to the research station, along forest trails or alongside the road from La Gamba to Golfito (Fig. 2).

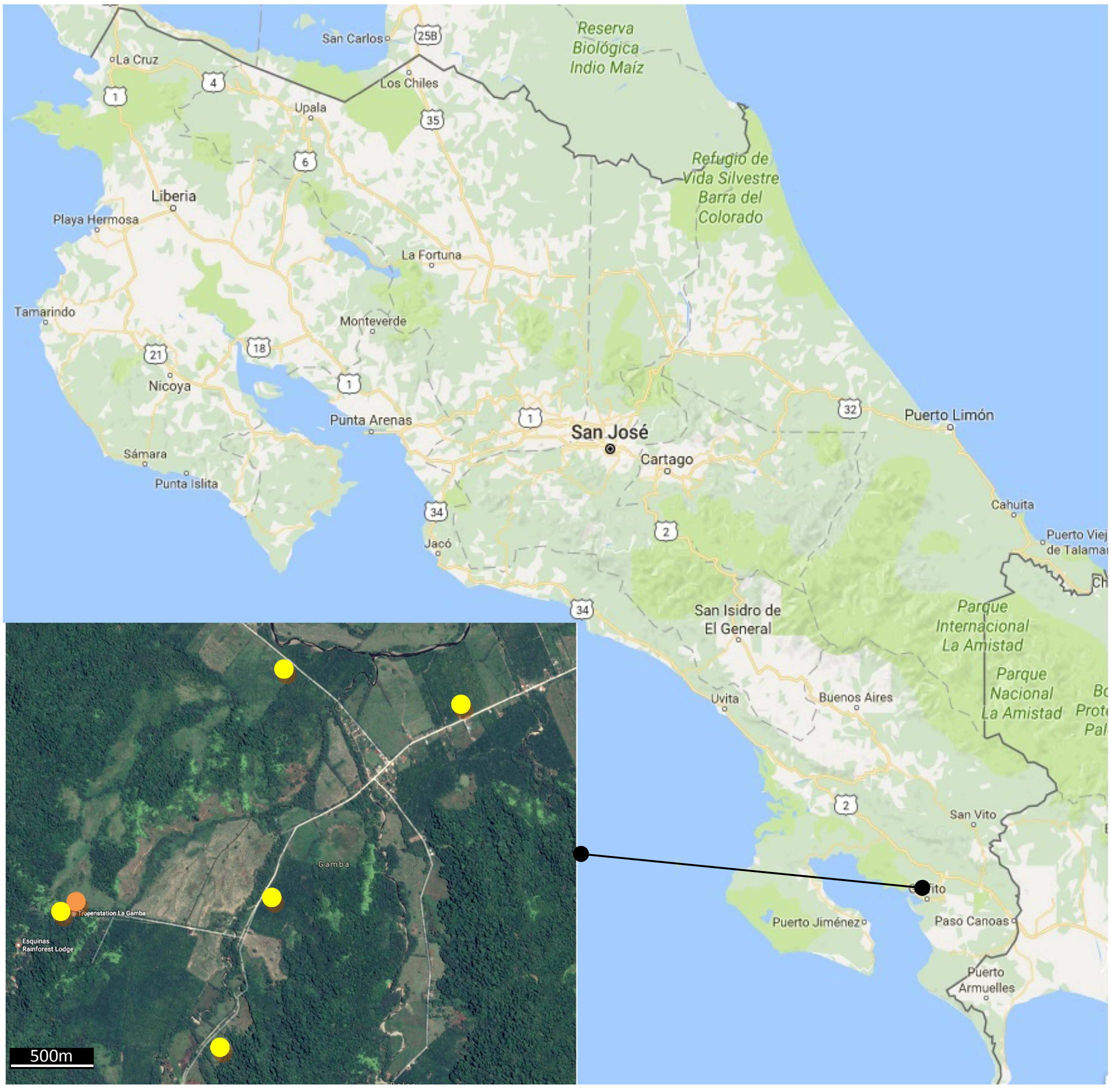

Fig. 2 Map of Costa Rica and close-up of sample collection area close to Golfito. All samples used in this study were collected (yellow dots marking collecting areas roughly, as no GPS data were taken) close to the research station (orange dot) in La Gamba, always along the roadside. (Modified 1.5.2017: https://www.google.at/maps/@8.6980452,-83.1916867,4356m/data=!3m1!1e3)

Collecting samples - with an insight into *Cecropia*-*Azteca* associations

In order to collect patch material, host-plants and inhabiting ants had to be identified. Three myrmecophytic *Cecropia* spp. (Urticaceae) can be found in the study region: *C. peltata*, *C. obtusifolia*, and *C. insignis*. These pioneer plants occur very often in recently opened gaps, along roadsides and abandoned fields (Kricher 1997). *Cecropia* has a very distinctive growth form (Fig. 3). The crown is formed by large, long-petioled, palmately-lobed, peltate leaves. Every leaf originates out of one individual young node. Older internodes lose their leaves but form the long, thin stem. Young internodes are filled with a soft pith, which retracts during development and remains as a thin layer lining the inner surface of the internodes wall, leaving the internode hollow to form domatia for ants. Septa between the internodes are formed by a hard, sclerenchymatic tissue (Bailey 1922, Longino 2005).

In Neotropical *Cecropia*-ant mutualisms are *Azteca* ants from the Dolichoderinae subfamily are the most common ant partners. Common *Azteca* species, in obligate associations with *Cecropia* trees are *A. coreuleipennis*, *A. alfari*, *A. constructor* and *A. xanthochroa* (Longino 1989, 2005).

After the nuptial flight, an ant queen selects a *Cecropia* sapling and enters a domatium by biting through a small area of unvascularized tissue, the so-called prostoma. The small hole is closed up again by the queen with tissue scraped off from the inside wall of the internode (Mayer *et al.* submitted). The queen is sealed off inside the internode, where she rears the first brood of workers. Before a single ant colony occupies one *Cecropia* tree, the internodes of the sapling may be occupied by many queens and their first brood (Choe and Perlman 1997, Longino 2005). Multiple-queen founding (pleometrosis) is common in *Cecropia-Azteca* associations as documented by Mayer *et al.* (submitted). Nevertheless, after a while only one *Azteca* colony with a single queen becomes dominant on any given *Cecropia* individual (Choe and Perlman 1997, Longino 2005, Mayer *et al.* submitted).

The first mature ant workers chew their way out through the prostoma and start collecting food bodies (Müllerian bodies) to feed them to the larvae. Müllerian bodies are produced by the plant in the trichomes, at the petiole base, and are up to 2mm long, white, and glycogen containing (Fig. 3B) (Rickson 1971, Bischof *et al.* 2013).

Hosted *Azteca* ants not only provide their plant with nitrogen through deposited dead ants, but also protect their *Cecropia* tree against herbivores and competing vegetation, like vines (Sagers *et al.* 2000, Rico-Gray and Oliveira 2007).

In addition, chaetothyrialean fungal patches were found in all *Cecropia* plants inhabited by *Azteca* ants, investigated by Nepel *et al.* (2016), but a benefit for the plant of the ant-tended fungal patches has not been proven yet.

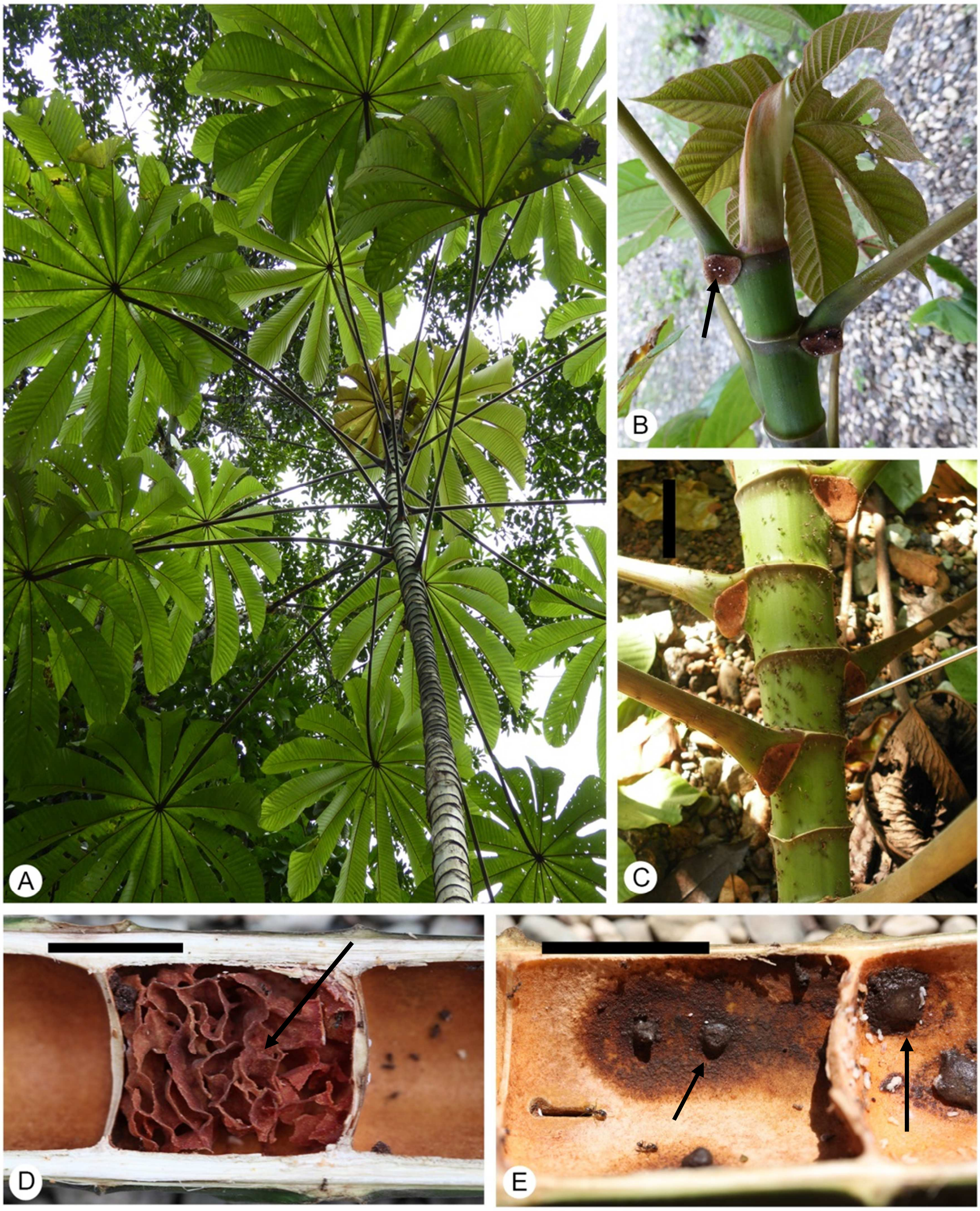

Fig. 3 Study plant. **(A)** Habit of *Cecropia obtusifolia* with peltate leaves. **(B)** Dense mat of trichomes (trichilia) at the base of the leaf petioles produce food bodies (so-called Müllerian bodies) (arrow). **(C)** Alerted ants on a *Cecropia* sp. stem. **(D)** Longitudinally opened *Cecropia* sp. stem, showing hollow internodes (domatia) with carton structure constructed by *Azteca constructor* (arrow). **(E)** Black patches (arrows) of organic material with fungal hyphae in the domatia are the main objects of interest in this thesis. Bars: (C) 4cm, (D) 2cm, (E) 2cm. Photos: Mayer V.

In order to collect fungal patches and carton structures, colonized *Cecropia* trees had to be found, cut down, and transported into the research station (Fig. 4). Then, the stems were opened up lengthwise using a machete and samples were gathered. Samples for this thesis comprised material from 50 different *Azteca* colonies. To achieve sequences of chaetothyrialean fungi, samples included carton material (Ec) and fungal patches (Ep) of inside the domatia. Six samples were taken of the leaf or stem surface of occupied plants for testing occurrence of chaetothyrialean fungi outside the domatia.

Fig. 4 Collecting samples. A cut *Cecropia* tree is loaded by V. Mayer for transport to the research facility.

Sample treatment

Azteca inhabited *Cecropia* trees (1-9m tall) were cut and their stems where opened lengthwise. Patch and carton material was collected with sterile forceps and stored separately in Eppendorf tubes. The tubes were closed with air permeable cotton wool and dried in an oven at 35 °C. The dried samples were then stored in a box filled with silica gel.

Extraction

DNA extraction was done with a Quiagen DNeasy® Plant Mini Kit (Quiagen, Hilden, Germany) following the manufacturers protocol. In the first step, small amounts of the samples, approximately 25mm², were filled into a 2mL tube, adding 3 to 5 beads (Ø 3mm) and ground for 5 min at 30Hz, using a TissueLyserII. Number of beads varied to fit the amount of sample used. The following steps were done according to the Quiagen Quick-StartProtocol, except for the volume of the final elution buffer (25µL were used instead of 100µL). After extraction, the lysate was stored in the freezer for further steps.

PCR

As marker for phylogenetic analyses, I chose a ribosomal DNA fragment containing the small subunit 18S (SSU), the complete ITS1-5.8S-ITS2 unit (ITS) and the large subunit 28S (LSU). In order to multiply the target DNA for sequencing, polymerase chain reaction (PCR) and semi-nested PCR were performed using an Eppendorf Mastercycler. For PCR 1µL lysate from the extraction containing the whole genome, was amplified with 9µL containing Thermo Scientific 2X ReddyMix PCR Master Mix (1.5 mM $MgCl_2$) (Thermo Fisher Scientific, USA), the forward primer V9G (De Hoog and Gerrits van den Ende 1998) and reverse primer chaeD-R (Nepel *et al.* 2016). The detailed PCR protocol started with an initial 3 minutes denaturation at 95 °C and was followed by 50 cycles of 15 seconds denaturation at 95 °C, 20 seconds annealing at 53 °C and 1 minute elongation at 72 °C. A final elongation step of 3 minutes at 72 °C was done before the ultimate cooldown to 15 °C. All PCR products were checked for clear bands with gel electrophoresis. If there was no clear band, a semi-nested PCR (snPCR) of 1µL PCR product was performed with another primer set, following the protocol as described above (S.1). For snPCR, instead of the forward primer V9G, the primer ITS5 was used because ITS5 binds closer to the end of the SSU region. The reverse primer chaeD-R generally amplifies the whole ITS2 region, but did not always result in clear bands; hence it was replaced with chaeDITS-R, which binds in the last 60bp of the ITS2 region (Fig. 5, S.2) and results in shorter fragments. All PCR products were stored in the freezer for further steps. After another check for bands with 1% agarose gel electrophoresis only the products showing bands were further analysed by Sanger cycle sequencing (Nepel *et al.* 2016).

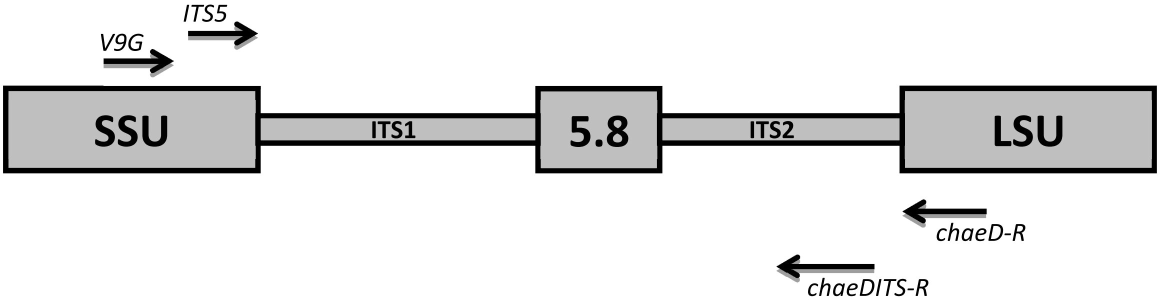

Fig. 5 Ribosomal DNA operon. Forward primer V9G and ITS5 binding in the small sub unit. Reverse primer chaeD-R binding in the large sub unit (LSU) and chaeDITS-R in the last 60bp of the internal transcribed space 2 (ITS2)

Sanger cycle sequencing

Before cycle sequencing, samples were purified using the Eppendorf Mastercycler. For purification 1µL containing ExonucleaseI and FastAP Thermosensitive Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) (Werle *et al*. 1994) at a ratio of 1:2 were added to each Eppendorf tube containing 8µL snPCR product. Protocol for purification started with 45 minutes incubation at 37 °C and was followed by a step of 15 minutes enzyme deactivation at 85 °C before the program ended with storage at 4 °C.

Cycle sequencing was performed on a GeneAmp® PCR System 9700 with 0,5mL Eppendorf tubes. For every snPCR sample two Eppendorf tubes were filled with 4µL snPCR product and 6µL Mastermix. Mastermix always consisting of 1,2µL ddH$_2$O, 2µL 5xBuffer (3.1), 2µL 1M Trehalose, 0,5µL Big Dye(3.1) (Thermo Fisher Scientific, USA) and 0,3µL forward or reverse primer. 6µL Mastermix, containing ITS5 forward primer, were added to one tube. 6µL Mastermix containing the same reverse primer according to the snPCR sample product (either ChaeDITS-R or ChaeD-R) were added to the other. Both were cycle sequenced. The protocol for cycle sequencing started with an initial 1 minute denaturation at 96 °C and was followed by 40 cycles of 10 seconds denaturation at 96 °C, 5 seconds annealing at 50 °C and 2 minutes elongation at 60 °C (Nepel *et al.* 2016). After the cycles the products were kept at 4 °C before further preparation.

Phylogenetic reconstruction

SeqMan and BioEdit

Resulting forward and reverse sequences were checked and overlaps produced with SeqMan Pro DNASTAR Lasergene (Version 7.7.0) program. All resulting amplicons were revised with BioEdit, Version 7.2.5 (Hall 1999) and embedded into a matrix, consisting of selected reference sequences from previous studies of the *Cecropia-Azteca* association (Nepel *et al.* 2016) and other ant-plant interactions (Voglmayr *et al.* 2011).

Clustered genotypes of the *Azteca* symbiotic chaetothyrialean fungi are described as species and were summed up in six individual operational taxonomic units (OTUs), each with 99% ITS similarity (Schoch *et al.* 2012, Nepel *et al.* 2016). The twelve genotypes, clustered in six OTUs, formed the base of phylogenetic reconstruction in this thesis. It needs to be pointed out that OTU6, which contained only one genotype, was recently assigned to OTU2 (Mayer *et al.* submitted).

The BioEdit matrix for OTU assignment includes sequences that had been gained in Nepel *et al.* (2016) and sequences of the so called 'domatia symbiont clade', described in Voglmayr *et al.* (2011). Figures showing affiliations were created with Microsoft® Office Excel 2010.

Paup

For phylogenetic reconstruction, maximum parsimony (MP) bootstrap analyses were performed with PAUP* version 4.0a152 (Swofford 2002). 1000 replicates of heuristic search with 10 rounds of random sequence addition each bootstrap replicate were conducted. The limit of rearrangements per replicate was set to 100 000. Additional parameters: TBR branch swapping, allowing multitrees; steepest descent was set to no. Gaps were treated as missing data and no weighting of nucleotides was applied. Maximum parsimony bootstrap analysis was conducted according to (Nepel *et al.* 2014). Trees were rooted with *Cladophialophora scillae* [EU035412] and *Cladophialophora hostae* [EU035407] as outgroups.

Morphological investigation of patches

Scanning electron microscopy (SEM)

For observation of fungal patches and carton with SEM, samples that were preserved in FAA (formaldehyde-acetic acid-alcohol) and stored in 70% ethanol, and one patch sample preserved with glutaraldehyde were used. All samples were dehydrated in an ethanol series with increasing concentration and acetone as final step. After dehydration they were critical point dried (CPD) with acetone as exchange fluid using an Autosamdri-815 (Tousimis Research Corporation, USA). CPD samples were mounted on stubs using double-sided adhesive tape or clear nail polish. They were sputter coated with gold in an argon atmosphere for 3min with a BAL-TEC SCD050 Sputter Coater. Afterwards, the samples were placed in a JEOL JSM – IT300 SEM (Nihon Denshi K.K., Japan) and observed at 10 or 20KV.

Light microscopy

For morphological investigation of fungal patches, a light microscope (Olympus BX50) was used and pictures were taken with a Nikon Digital Sight DS-Fi1 camera. Since all material of sequenced patches was already used up, other samples of fungal patches and carton material of *Cecropia-Azteca* associations, which had been collected and stored in the same way, were chosen for morphological investigation.

In order to soften the material, a very small amount of dried sample material was placed in 2mL Eppendorf tube, filled with water and left to soak. After two hours, samples were vortexed and a droplet of water with patch material was put on a microscope slide. In order to gain well separated particles the material was torn apart with tweezers and needles. Afterwards, a cover slip was placed on the water drop and the sample was slightly squeezed before observation with the light microscope. For better contrast some of the fungal hyphae were stained with Lactophenol blue solution (Sigma Aldrich, USA).

Results

Morphology of patches

Light microscopy

Investigation of environmental fungal material with light microscopy showed a vast diversity of different components included in the material: unidentified organic material, hyphae, fungal spores (Fig. 6F), nematodes and nematode eggs. Whereas substrate hyphae are hyaline (Fig. 6H), conidiophores are brownish with melanin in the cell walls (Mayer *et al.* 2009) (Fig. 6A). In addition, erect conidiophores were observed (Fig. 6A, D). Apart from fungi, debris and other tissue, also nematodes (Fig. 6C) were found in nearly all samples of fungal patches, but not in carton samples. While fungal patch samples were straightforward to process, carton samples did not soften enough to be easily handled. When the material was not torn apart enough, observation in the light microscope revealed only brown clumps. Samples stained with Lactophenol blue solution resulted in blue nematodes only. Staining of fungal hyphae was not achieved which may be due to the melanin incrustations of the hyphal cell walls.

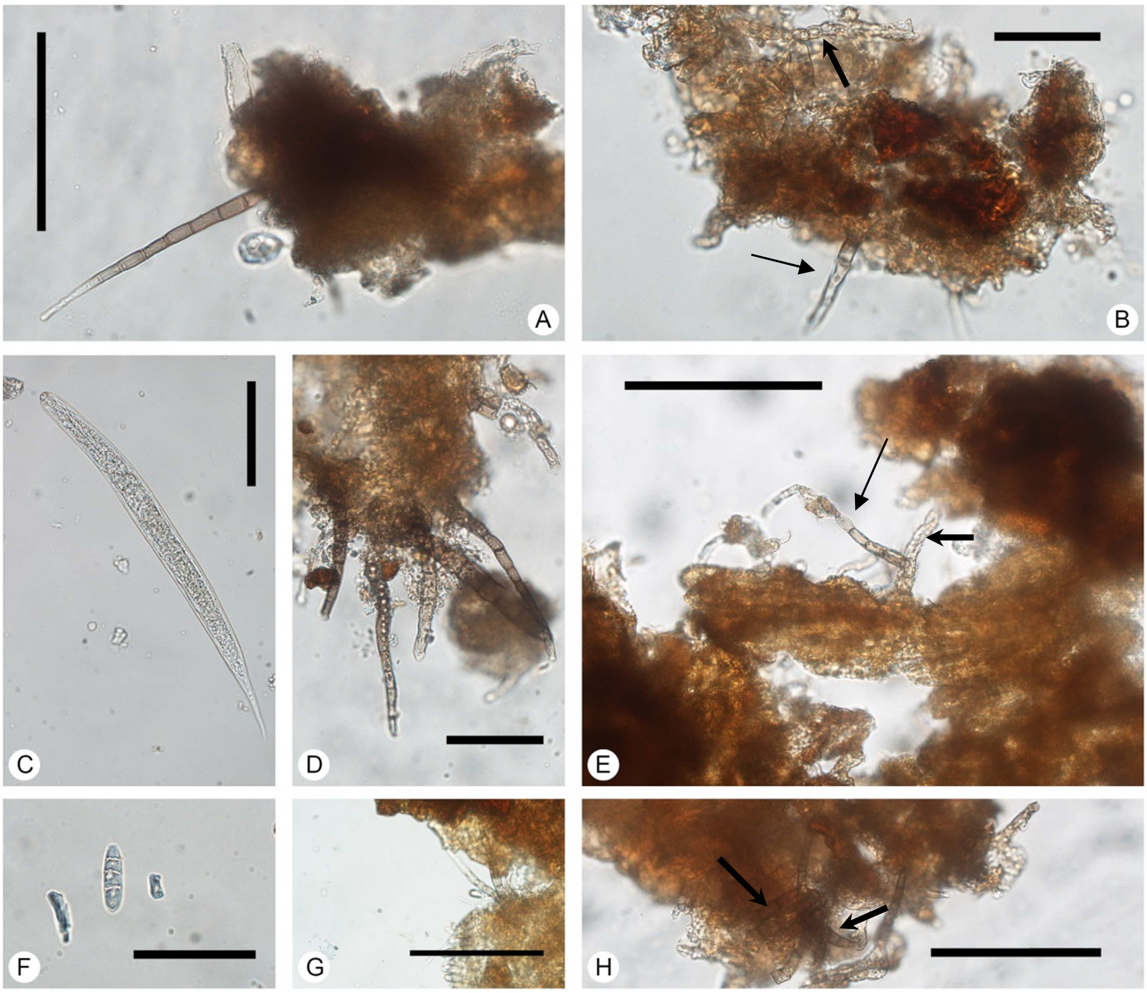

Fig. 6 Fungal patches of *Cecropia peltata* in association with *Azteca constructor* with their different components. **(A)** A fungal conidiophore, **(B, E)** hyaline substrate hyphae (➞) and conidiophores (⟶) of domatia fungi, **(C)** Nematode, **(D)** Conidiophores, **(F)** Conidia, **(G)** Conidiophore with conidia, **(H)** substrate hyphae (arrows). Bars: (A, C, E, G, H) 100µm, (B, D, F) 50µm.

SEM

The carton structures seem to have a relatively homogeneous and smooth surface (Fig. 7A). Higher magnifications reveal fungal hyphae partially or wholly embedded in an amorphous matrix (Fig. 7B, C). Figure 7C, 7D and 7E show the fungal hyphae in detail. Note, that there were no nematodes in carton samples.

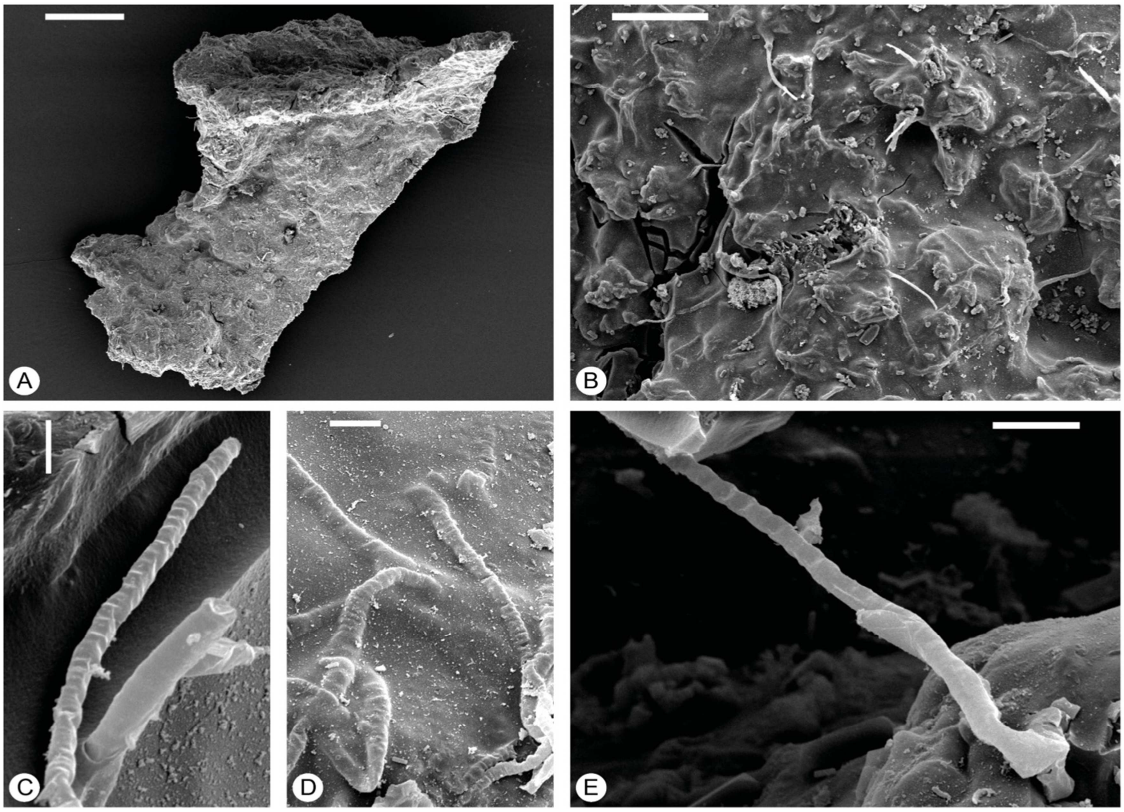

Fig. 7 Carton from domatia of a *Cecropia obtusifolia* and *Azteca constructor* association. **(A)** Overview of carton surface. **(B)** Close up on the surface, **(D)** hyphae growing tight under carton surface and **(C, E)** stretching out into the air. Bars: (A) 500µm, (B) 100µm, (C) 5µm, (D, E) 10µm.

In fungal patches preserved with glutaraldehyde, precipitations are seen, but higher magnifications reveal fungal hyphae quite clearly. The precipitations formed during preparation process. A network of chaetothyrialean fungal hyphae can be seen in Fig. 8C. In the close-up Fig. 8D, E, hyphae seem collapsed, which may be due to the glutaraldehyde treatment.

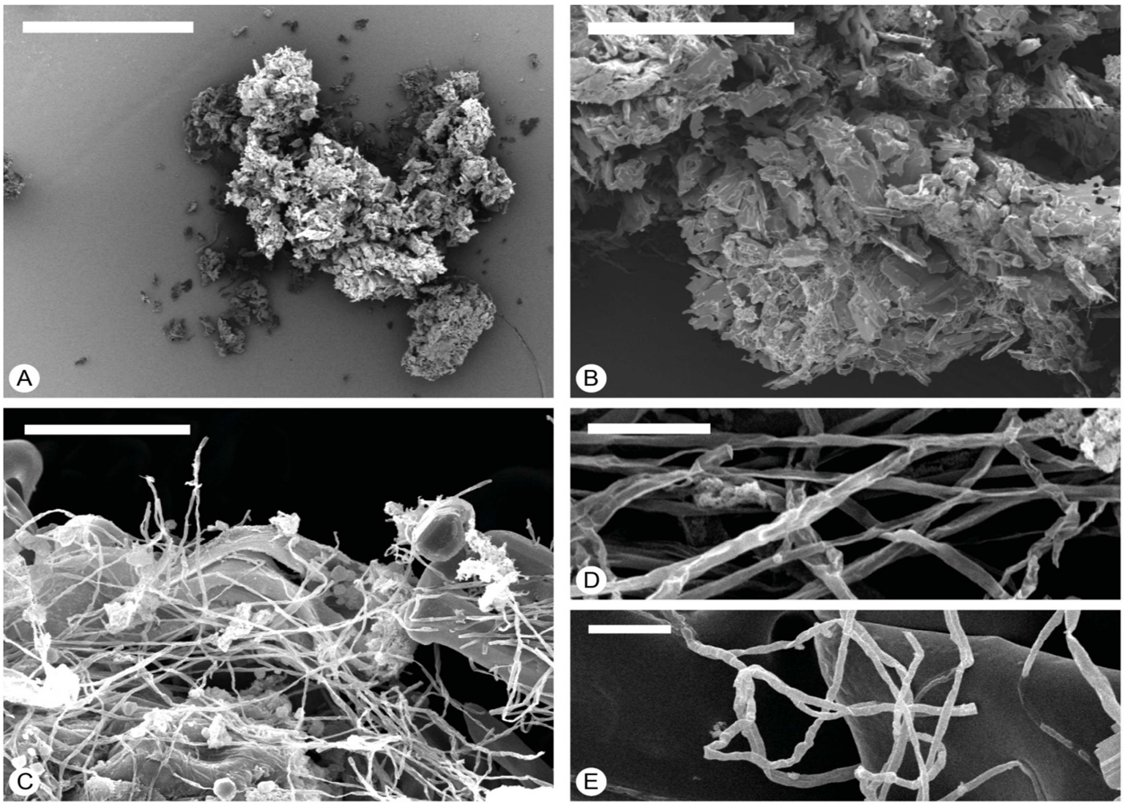

Fig. 8 With Glutaraldehyde preserved fungal patch samples. **(A)** Overview of crystallized sample. **(B)** Alien looking surface of the patch sample. **(C)** Fungal hyphae forming a superficial net. **(D, E)** Seemingly dried out hyphae branching off and intermingled. Bars: (A) 2mm, (B) 500µm, (C) 50µm, (D, E) 10µm.

Another sample of a fungal patch with a rough surface can be seen in Fig. 9A. It reveals a cluster of plant fibres and ant debris (Fig. 9B). Parts of the exoskeleton of dead ants are enclosed in the patch material (Fig. 9D). Nematodes are abundant throughout the whole sample and can be seen between ant debris and other patch particles (Fig. 9E).

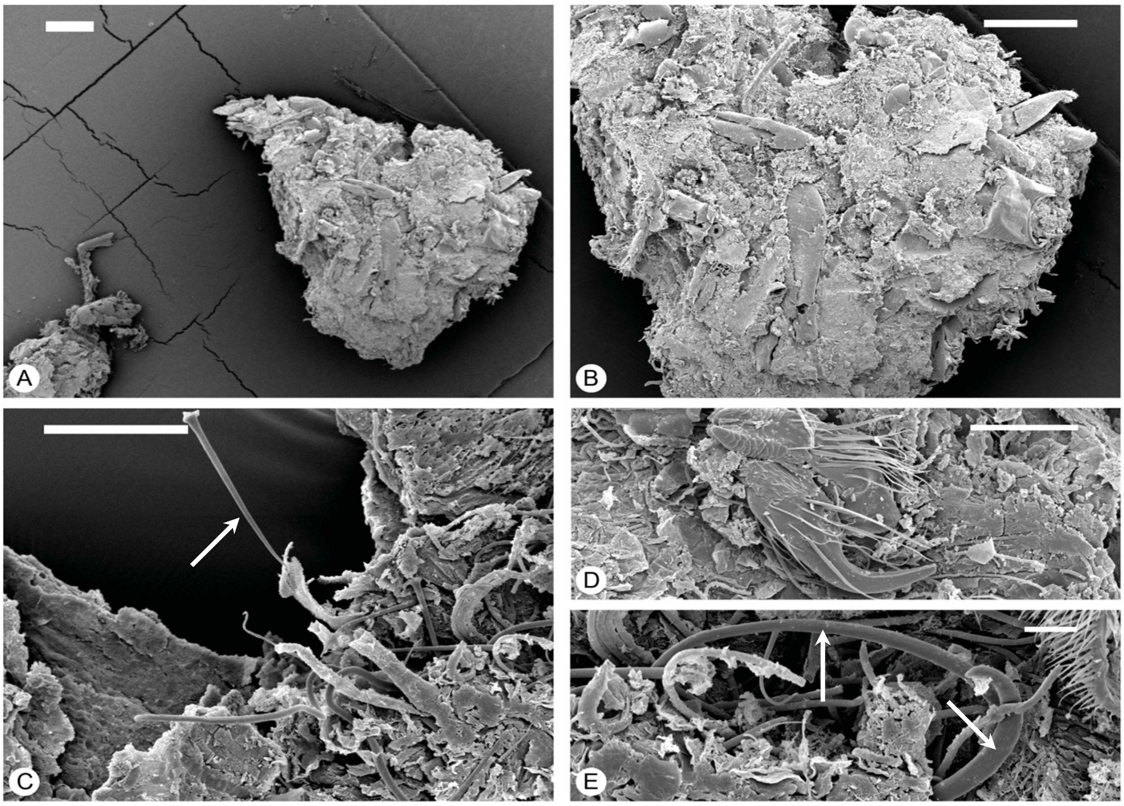

Fig. 9 Fungal patch of a *Cecropia obtusifolia* and *Azteca constructor* association. **(A)** Overview of patch sample. **(B)** Close up showing a lot of ant debris enclosed in the sample. **(C) A** trichome (arrow) from the *Cecropia* stem and other components. **(D)** Parts of the exoskeleton of dead ants. **(E)** Nematodes in patch material (arrows). Bars: (A) 2mm, (B) 500µm, (C) 50µm, (D, E) 10µm.

Molecular phylogenetic analyses

Phylogram and OTU assignment

Three out of the 5 (previously 6) known *Azteca* associated operational taxonomic units (OTUs) were present with at least one genotype in the set of samples analysed in this study (Table 1). Surprisingly, the sequence of sample no. 65 (16Cec19 EcIII const) is most closely related with a chaetothyrialean fungus found in the Southeast Asian *Cladomyrma-Saraca* association [MACP1]. Obviously, sequences of *Cladomyrma-Saraca* associations are not assigned to any OTU of a *Cecropia-Azteca* association. As a result, the assigned reference for No. 65 is titled with 'CS' instead of any OTU in Table 1 and the following figures. From the six samples taken of leaf and stem material, only three were with chaetothyrialean sequences.

The matrix contained 37 different chaetothyrialean genotypes. By adding representative sequences from Nepel *et al.* (2016) (n=33), Voglmayr *et al.* (2011) (n=10), Mayer *et al.* (submitted) (n=20) and Genbank (n=2), the final alignment consisted of 114 sequences with 473 alignment positions. 96 characters were parsimony-informative. The resulting best tree out of 407101 after heuristic search with 10 replicates served as base for Figure 10. Bold branches indicate Maximum parsimony bootstrap support above 80 %. Similar to the resulting tree of Nepel *et al.* (2016), all sequenced Chaetothyriales geonotypes added in this study also belong to OTUs defined by Nepel *et al.* (2016) of the domatia symbiont clade, originally described in Voglmayr *et al.* (2011).

Table 1 shows the distribution of OTU assigned sequences of the 37 chaetothyrialean strains of this study, and their affiliation to the subordinated genotypes.

OTU1 with the genotype 'Cec7c' was present in one sample (No. 39 - 16Cec21_EcIII_alf). OTU2 was present in fourteen samples with three different genotypes. OTU3 was found in 21 sequences, and all affiliated with the genotype 'chaeD-CR-7'. No sequences out of the samples used for this study were assigned to any genotype belonging to OTU4 or OTU5. For the sample No. 65 (16Cec19 EcIII const), from an *A. constructor* and *Cecropia peltata* association, CS for the sister to *Cladomyrma – Saraca* [MACP1] is given instead of an OTU.

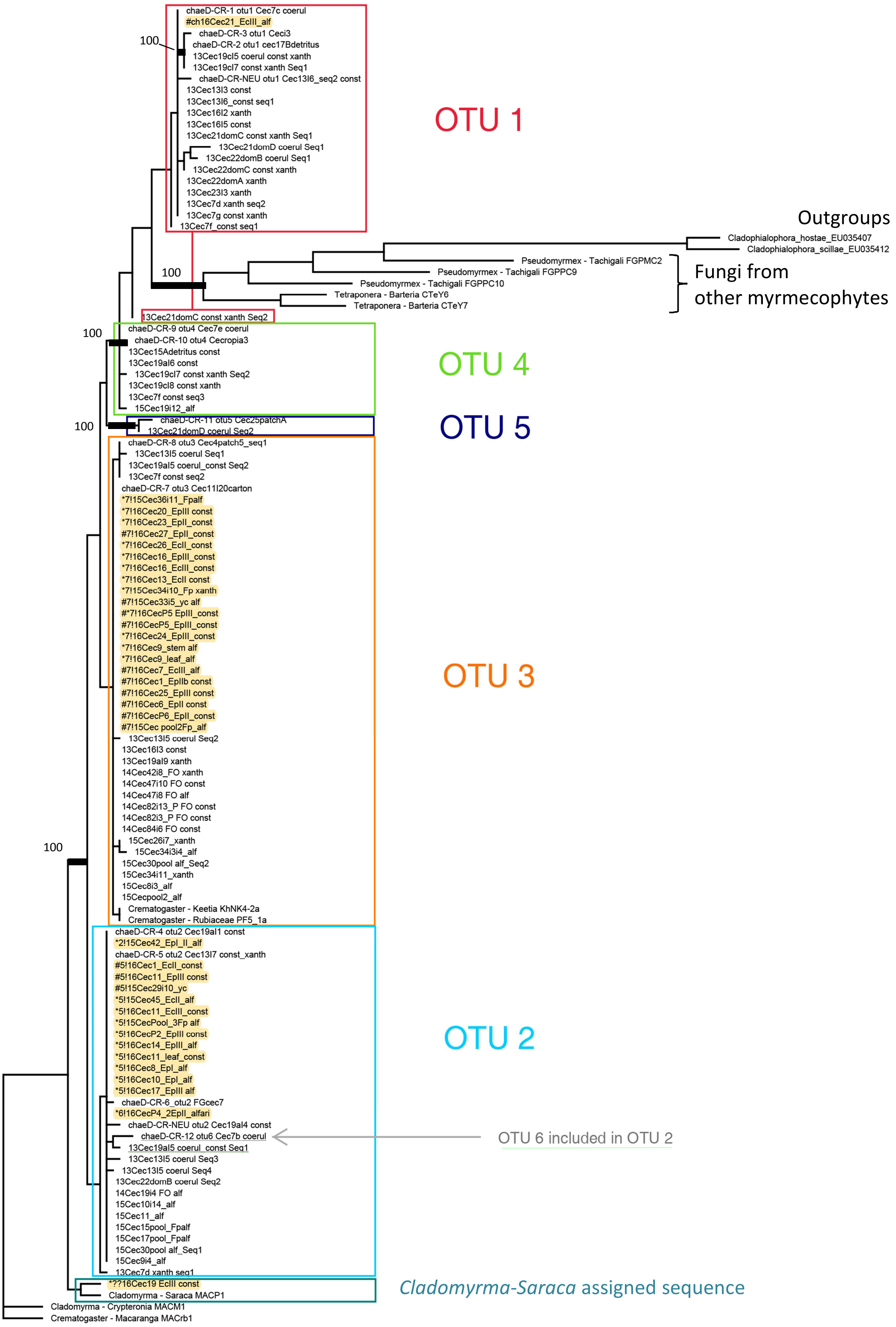

Fig. 10 The best fitting phylogram after MP analysis of ITS of chaetothyrialean rDNA, gained from domatia fungi. *Cladophialophora* species are used as outgroup. The sequences produced in this study are assigned to OTUs suggested by Nepel *et al.* (2016) and are framed in colours. All 37 sequences gained in this thesis are highlighted with yellow. Bold branches indicate bootstrap support >80%.

Tab. 1 Affiliation of analysed sequences. Chaetothyriales strains investigated from *Cecropia-Azteca* associations with OTU and genotype assignment. As No. 65 was assigned to a Southeast Asian plant-ant association affiliation is given as CS[MACP1].

			OTU1	OTU2			OTU3
No.	**Sample-ID**	***Azteca* species**	chaeD-CR-1 Cec7c	chaeD-CR-6 FGCec7	chaeD-CR-5 Cec14I7	chaeD-CR-4 Cec19al1	chaeD-CR-7
1	16CecP4_2EpII	*alfari*		x			
2	ch16CecP5_EpIII	*const*					x
5	16Cec24_EpIII_const	*const*					x
6	16Cec25_EpIII const	*const*					x
9	ch16CecP5_EpIII_1	*const*					x
10	16CecP6_EpII_const	*const*					x
13	16Cec10_EpI_alf	*alfari*			x		
14	16Cec11_EpIII const	*const*			x		
15	16Cec11_EcIII_const	*const*			x		
17	16Cec11_leaf_const	*const*			x		
18	16Cec6_EpII const	*const*					x
19	16Cec9_stem alf	*alfari*					x
20	16Cec9_leaf_alf	*alfari*					x
21	16Cec7_EcIII_alf	*alfari*					x
23	16Cec1_EcII_const	*const*			x		
24	16Cec8_EpI_alf	*alfari*			x		
25	16CecP2_EpIII const	*const*			x		
27	16Cec1_EpIIb const	*const*					x
32	16Cec14_EpIII_alf	*alfari*			x		
33	16Cec16_EpIII_const	*const*					x
34	16Cec16_EcIII_const	*const*					x
35	16Cec17_EpIII alf	*alfari*			x		
38	16Cec20_EpIII const	*const*					x
39	ch16Cec21 EcIII	*alfari*	x				
40	16Cec23_EpII_const	*const*					x
42	16Cec27_EpII_const	*const*					x
46	15Cec42_EpI_II_alf	*alfari*				x	
50	15Cec34i10_Fp xanth	*xanth*					x
51	15Cec36i11_Fp alf	*alfari*					x
52	15Cec pool2Fp_alf	*alfari*					x
53	15CecPool_3Fp alf	*alfari*			x		
54	15Cec29i10_yc	*YoungCol*			x		
57	15Cec33i5_yc alf	*alfari*					x
60	15Cec45_EcII_alf	*alfari*			x		
62	16Cec13_EcII const	*const*					x
64	16Cec26_EcII_const	*const*					x
65	16Cec19 EcIII const	*const*	CS[MACP1]				

OTU distribution and ant species

From the 37 sequences, 20 samples were from *A. constructor* colonies, 15 from *A. alfari* colonies, one from an *A. xanthochroa* foundress queen fungal patch and one from a young colony (no ant species given).

The chaetothyrialean fungi found in the samples analysed here could be assigned to three different OTUs. 38% of all samples were assigned to OTU2 and 56% to OTU3, 3% to OTU1 and 3% to none of the known *Cecropia-Azteca* OTUs (Fig. 11).

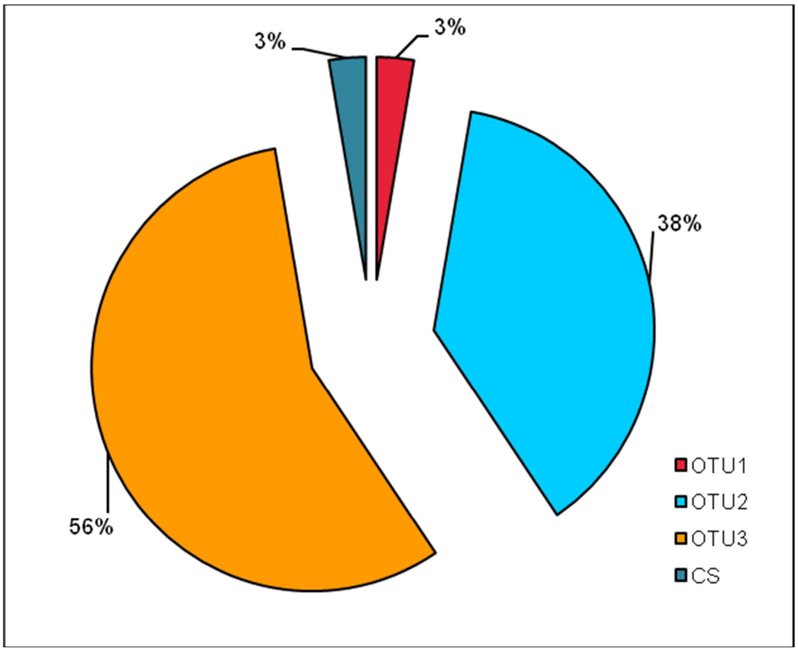

Fig. 11 Assignment of all sequences analysed in this study (n= 37) to OTUs defined in Nepel *et al.* (2016) in percent. OTU2 and 3 are the dominant ones.

Sequence assignments to OTU2 and 3 of samples of *A. alfari* and *A. constructor* colonies show that different *Azteca* species seem to prefer different OTUs of chaetothyrialean fungi (Fig. 12).

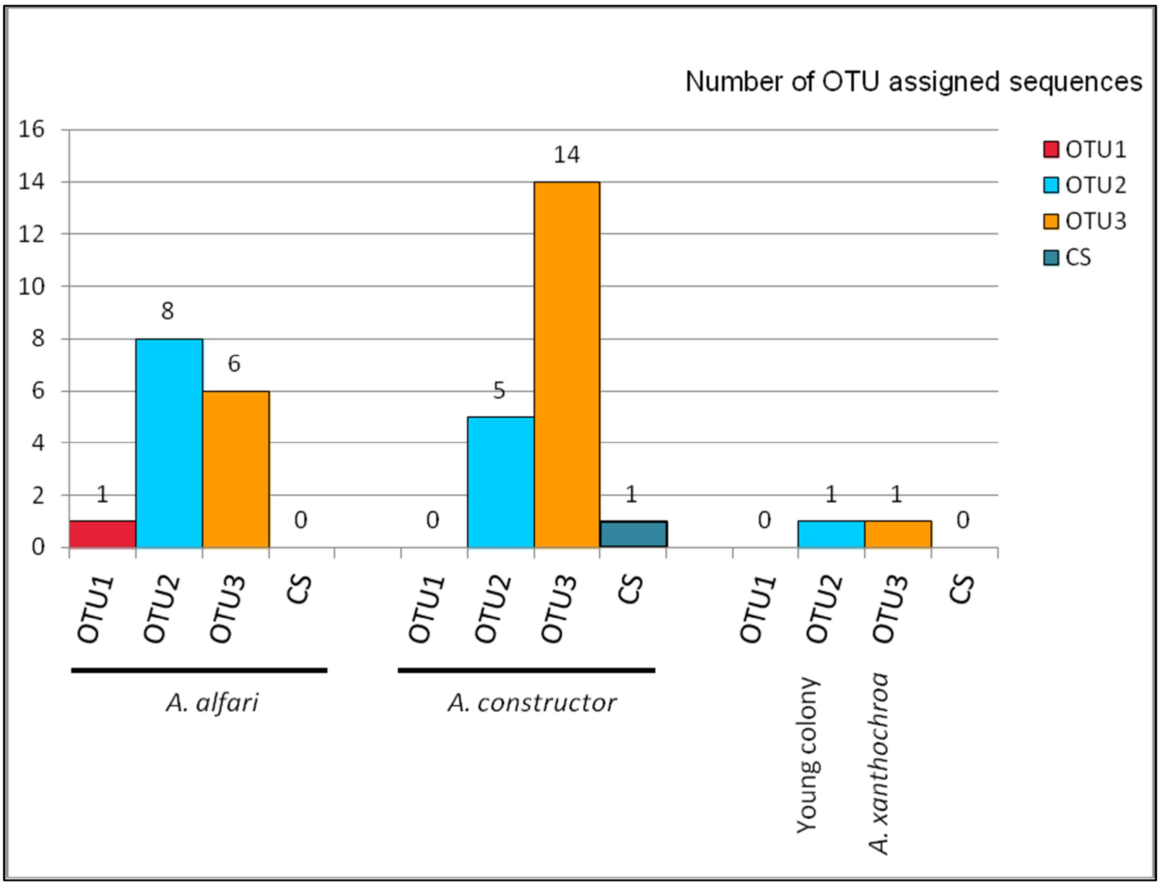

Fig. 12 Number of OTU assigned sequences. In colonies of *Azteca alfari* (n=15), *A. constructor* (n=20), of a young colony of an unidentified ant species (n=1) and an *A. xanthochroa* queen (n=1)

14 sequences out of 20 of the *A. constructor* group were assigned to OTU3, 5 to OTU2 and one was sister to *Cladomyrma* - *Saraca* [MACP1] (Fig. 13A). Eight sequences out of 15 of the *A. alfari* group were assigned to OTU2, six to OTU3 and one was assigned to OTU1 (Fig. 1B).

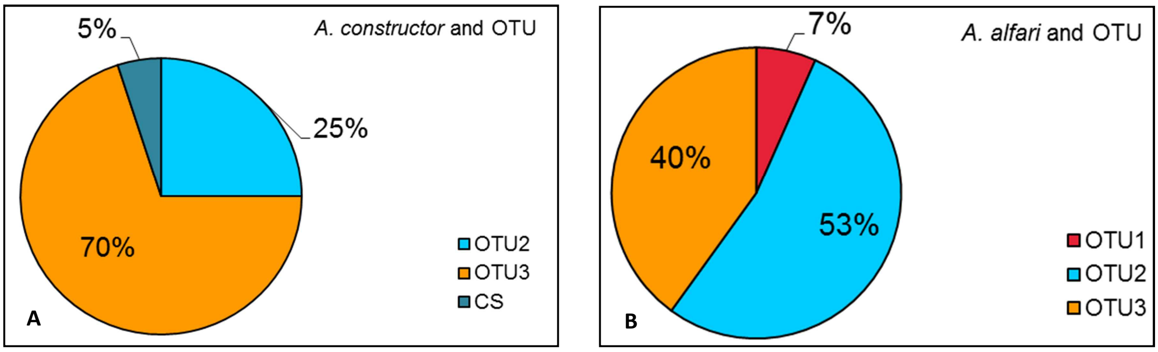

Fig. 13 *Azteca* sp. and OTU assignments. **(A)** Assignments of *A. constructor* (n=20). **(B)** Assignments of *A. alfari* (n=15).

Discussion

Results of morphological investigation and molecular analysis of chaetothyrialean fungi from Cecropia-Azteca associations show that much is still unknown about this multi-mutualism. Whereas morphological investigations could add to the knowledge of the function of the hyphae within the mutualisms, further molecular studies might eventually result in the finished phylogenetical puzzle of chaetothyrialean fungi. Combined results might then give hints on ant-plant-fungi coevolution.

Morphologically, the most prominent differences between fungal patches and carton structures found inside domatia are not the fungal hyphae themselves, but the additional occurring components. These were made visible with light microscopy and scanning electron microscopy. The presence of nematodes in fungal patches and their absence in 'carton' samples initiate thoughts about the role of the nematodes in the patches, which has not been a subject of studies yet. Also, the initial role of the fungi inside the patches themselves, has not been well-defined so far. The interior 'carton' can be compared to exterior structures, like ant made carton nests and runway galleries, indicating similar stabilizing function for the hyphae. Although a resemblance of exterior and interior carton structures is given, carton inside domatia show less diversity in fungal hyphae than external carton, when analysed molecularly (Nepel *et al.* 2016). It seems that the fungal patches, containing microorganisms and deposited ant debris, are a complex microbiome and the chemical process within, needs yet to be characterized.

Although Chaetothyriales are weak competitors in generally favourable environments (Zhao *et al.* 2010), they seem to thrive, where other fungi cannot exist if they are tended by specific ant species. Since ascomycetes have been identified as symbionts of *Lasius* ants in the walls of their carton nests (Schlick-Steiner *et al.* 2008), knowledge about ascomycetous ant symbionts has increased strongly. Especially fungi of the ascomycetous order Chaetothyriales seem to be ubiquitous, wherever ant-carton structures or ant-occupied domatia can be found (Voglmayr *et al.* 2011, Vasse *et al.* 2017). Ants produce toxic antifungal and antibacterial compounds in their glands, which can be tolerated and metabolized by chaetothyrialean fungi (Schlüns and Crozier 2009, Voglmayr *et al.* 2011). Hence, this ascomycete order is highly pre-adapted for occupying various ant-symbiont niches (Nasciemento *et al.* 2016, Vasse *et al.* 2017).

All 37 sequences gained from leaf and stem material, fungal patches and ‘carton’ material of domatia from *Cecropia-Azteca* associations in this study, were assigned to OTUs of the “domatia symbiont clade” defined in Voglmayr *et al.* (2011). They were assigned to three OTUs described in Nepel *et al.* (2016) and one sequence was assigned to a South Asian ant-plant-fungi association. Hence, the hypothesis that the domatia samples would be characterized by low genotype and OTU diversity could be confirmed. The preference for specific OTUs by different *Azteca* ants can be approved for *A. alfari* and *A. constructor*. Let alone results of the present study, combined with the results for OTU preference by *A. alfari* and *A. constructor* of Nepel *et al.* (2016), even clearer outcomes can be seen. Figure 14A shows the preference of *A. alfari* for OTU2 with 53% in 28 samples. In 33 samples of *A. constructor* and fungi symbioses, 61% were assigned to OTU3 (Fig. 14B). Since there was only one sample of a *Cecropia* sp. association with *A. xanthochroa* and none of *A. coreuleipennis* in the present study, conclusions regarding OTU preferences for those ant species can only be made vaguely. If more analysed samples would follow the given trend in Fig. 15, which shows OTU assignments of fungal sequences of four *Azteca* species, a preference of *A. xanthochroa* for OTU3 and OTU1 should be the outcome. No clear presumptions for any OTU preferences of *A. coeruleipeinnis* can be given here, as the number of analysed samples is too low.

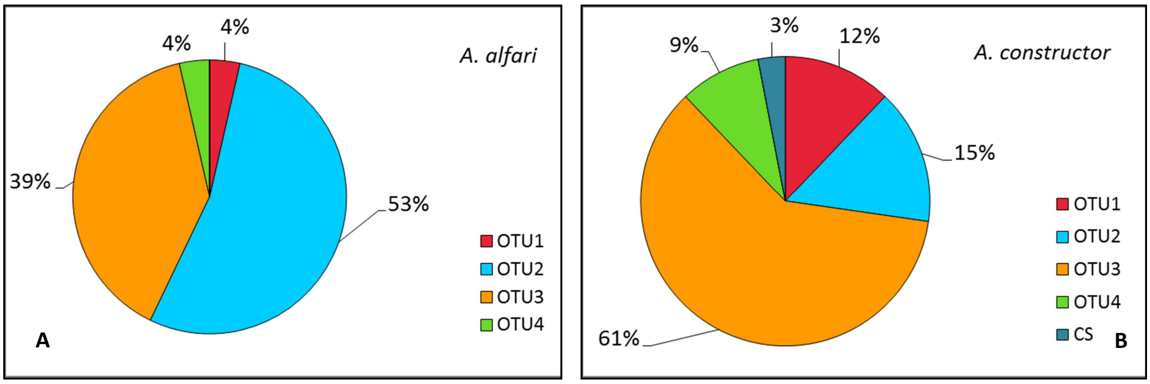

Fig. 14 OTU assignments including results from Nepel *et al.* (2016) and the study at hand, for **(A)** *A. alfari* (n=28) and **(B)** *A. constructor* (n=33).

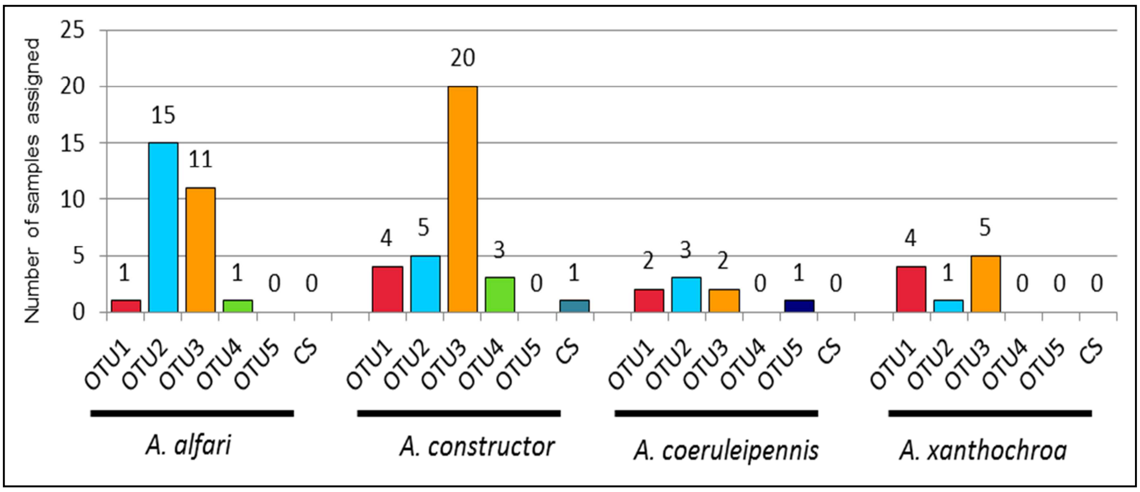

Fig. 15 OTU assignments for different *Azteca* species. Numbers include results by Nepel *et al.* (2016) and the study at hand.

Concerning the gained sequences of leaf and stem material, it is more likely that the surface was contaminated by the host ants. A higher sample number should be investigated to clarify this. Inevitably, considerations about why only a few genotypes can be found inside domatia and how they are transmitted initially are brought up and demand further investigations. Also, the confirmed ant-OTU preferences of *A. constructor* and *A. alfari*, suggest vertical fungi transmission by young queens.

The quantity of chaetothyrialean genotypes varies greatly between carton runway structures and fungal patches (along with 'carton' constructed inside domatia). Furthermore, it remains questionable, whether ants prefer specific chaetothyrialean fungi in exterior carton structures. An analysis by Nepel *et al.* (2014) shows 128 individual genotypes in constructed carton galleries on 18 different trees of *Tetrathylacium macrophyllum* (Salicaceae), and there seems to be no preference of *Azteca brevis* for a specific genotype. In contrast, given the results of the study at hand, combined with those of the first molecular study on *Cecropia*-*Azteca*-Chaetothyriales associations by Nepel *et al.* (2016), *A. alfari* shows clear preferences for one specific OTU in fungal patches. Furthermore, given preferences of *A. constructor* for one specific OTU of the study at hand, give another example of *Azteca*-Chaetothyriales affiliation. Hence, there is a big difference in chaetothyrialean genotype diversity, when comparing runway galleries on the plants' surface with 'carton' and fungal patches inside the domatia. This difference might be a result of the initial material used by the ants for starting either exterior or interior carton constructions and fungal patches.

Conclusions regarding the origin of the fungal hyphae in runway galleries can be drawn from results of a field experiment described by Mayer and Voglmayr (2009). For their arboreal structures, *Azteca brevis* ants use environmental material, like bark from the host tree, shredded epiphylls, small particles of epiphytes and, as shown in the experiment, even shredded fibres of adhesive tape if available. Soon after construction, the carton of runway galleries is covered and interspersed by a diversity of chaetothyrialean hyphae (Mayer and Voglmayr 2009). High genotype numbers and used material for construction of exterior carton suggest that fungi in galleries of *A. brevis* are 'either transmitted horizontally or environmentally acquired', according to Nepel *et al.* (2014). Spores or hyphal fragments can be transferred into runway galleries and arboreal carton nests along with the available organic material close-by, as described for fungi-growing termites by Korb and Aanen (2003). If this is the case, stabilizing fungal hyphae are not ant-specific, as already assumed by Vasse *et al.* (2017). In contrast to arboreal structures, the scraped-off masticated parenchyma material inside domatia, which is used for constructing interior 'carton' by *A. constructor* and *A. xanthochroa* (Nepel *et al.* 2016), has most likely never been exposed to the environment. Hence, I suggest that specific fungi are selected by ants either from the outside or, which I think is more likely, from the patch material inside the domatia. The question on the function of the hyphae inside the patches remains unanswered, but suggestions on how fungal patches are started can be made, yet the very first stages of fungal patch formations have not been clearly documented.

A theory on the vertical transmission of fungi for interior usage is based on better investigated ant-fungi relations, like *Atta* and *Lasius*. If the symbiosis of Chaetothyriales and *Azteca* ants, occupying *Cecropia* trees, is similar to the one of attini ants with basidiomycetes, the fungal patches inside of domatia might be started with fungal parts brought in by the queen. After the nuptial flight, Attini ant queens carry mycelia parts from their mother nest in their infrabuccal pocket and spit it out inside the freshly dug out chamber, in order to start a new fungal garden for their new colony (Huber 1905, Currie and Stuart 2001, Baker 2015, Hölldobler and Wilson 1990, 2016). Also, for the Old World *Lasius* ants, vertical transmission of the ascomycete fungi symbiont across generations was suggested by Schlick-Steiner *et al.* (2008), although the initial transmission of fungi into the new nest was not observed. Nevertheless, the behaviour of *Azteca* ants and the ant-specific OTU assignments found in the present study would support suggestions on vertical fungi transmission.

The investigation of fungi occurring inside the domatia of *Cecropia-Azteca* associations give directions for further studies, in order to understand the function of the fungi as well as a possible aspects of ant-fungi coevolution.

Conclusion

In summary, I could clearly show in this study that *Azteca constructor* and *Azteca alfari*, associated with *Cecropia* trees in Costa Rica, have preferences for certain chaetothyrialean OTUs. This is a strong argument for the hypothesis that fungi present in fungal patches are not acquired de novo for each colony founding, but rather are vertically transmitted from mother to daughter colonies. More samples from all tropical zones will be needed in order to complete the seemingly great puzzle of combined ant-plant-fungi-co-evolution.

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Zusammenfassung

Die *Cecropia-Azteca*-Vergesellschaftung ist schon lange ein klassisches Modell-System für Pflanzen-Ameisen Interaktionen. Die Wirtspflanzen stellen Wohnraum in Form von hohlen Stängeln (Domatien) und nährstoffreiche Futterkörperchen (Müllersche Körperchen) zur Verfügung, die Ameisen verteidigen „ihre“ Pflanze gegen Fraßfeinde. Aber neben der Pflanze und den Ameisen selbst, spielen auch Schildläuse, Nematoden, Bakterien und – erst kürzlich entdeckt – auch Pilze eine funktionelle Rolle. Diese Pilze gehören zur Ordnung Chaetothyriales (Schlauchpilze - Ascomycota) und kommen in sehr diversen, oft extremen Umgebungen vor. In Verbindung mit pantropisch verbreiteten, arborealen Ameisen, sind diese Pilze, welche zu den „schwarzen Hefen“ gehören, omnipräsente Mikroorganismen. Fast alle sind neu für die Wissenschaft und bisher noch nicht als Arten beschrieben worden. Die Pilzhyphen sind in den Domatien, sowie an externen Nest- und Tunnelbauten verschiedener Ameisenarten zu finden, wo das Geflecht der Pilzhyphen zur Stabilisierung der Wände beiträgt. In solchen externen, kartonähnlichen Konstrukten findet sich eine hohe Diversität verschiedener „Pseudoarten“ aus der Ordnung der Chaetothyriales (als „operational taxonomic unit“, kurz OTU zusammengefasste Genotypen). Weitaus weniger OTUs als in externen Kartonkonstrukten wurden bislang innerhalb der Domatien tropischer Ameisenpflanzen (Myrmekophyten) gefunden. Etwa fingernagelgroße „Pilzflecke“ werden in den Domatien von den Ameisen rege umsorgt. Auch im hohlen Stamm des neotropischen

Baumes *Cecropia* sp. werden solche Pilzflecken und, von Pilzhyphen durchzogener „Karton“, von Ameisen der Gattung *Azteca* sp. gehütet.

Um das phylogenetische Puzzle der Chaetothyriales zu vervollständigen, stehen derzeit Forschungen zur Diversität der Ameisen-Chaetothyriales im Fokus. Mit molekularen Methoden wurde in der hier präsentierten Studie die Diversität der Genotypen und OTUs der Pilze bei *Cecropia-Azteca*-Vergesellschaftungen untersucht. Auch etwaige Präferenzen verschiedener *Azteca*-Arten, insbesondere *A. constructor* und *A. alfari*, für bestimmte Pilz-OTUs konnten so erfasst werden. Ein Phylogramm der erhaltenen Sequenzen wurde unter Einbeziehung bereits bekannter Chaetothyriales-Sequenzen erstellt.

Chaetothyriale DNA konnte aus 37 von insgesamt 65 Pilzproben gewonnen werden. Durch Sequenzierung der teilweisen 18S (SSU), der kompletten ITS1-5.8S-ITS2 unit (ITS) und Teile der 28S (LSU) der ribosomalen DNA, konnten die in den Proben gefundenen Pilze OTUs zugeordnet werden. Alle Genotypen fanden sich in einem clade, bestehend aus “Domatien-Pilzen” die in Domatien von Ameisenpflanzen aus den Tropen weltweit gefunden wurden. Insgesamt wurden Vertreter aus nur 3 OTUs gefunden, womit die Hypothese, dass in Domatien der *Cecropia* eine geringe Diversität chaetothyrialer Pilze vorkommt, bestätigt wurde. 70% der Pilzsequenzen von *A. constructor* stammten aus der OTU3, 25% aus der OTU2 und eine Sequenz wurde in die Verwandtschaft eines Genotyps aus einer Süd-Asiatischen Ameisen-Pflanzen-Pilz-Vergesellschaftung eingeordnet. Dies spricht für eine kontinentübergreifende Verbreitung ameisenspezifischer Pilzarten aus der Ordnung der Chaetothyriales. 53% der *A. alfari* Proben wurden der OTU2, 40% der OTU3 und eine Sequenz wurde der OTU1 zugeordnet. Eine Präferenz für beide *Azteca* Arten ist somit gegeben.

Die geringe Diversität in den Cecropia-Domatien und die Präferenzen der Ameisen für einen bestimmten Pilz lassen auf Übertragung der Pilze von einer bestehenden Kolonie auf eine neugegründete Kolonie durch ausfliegende junge Königinnen schließen.

Die hier präsentierte Studie liefert einen weiteren Teil, der dem Gesamtpuzzle der Ameisen-Pflanzen-Pilz-Vergesellschaftungen hinzugefügt werden kann. Zu dessen Vervollständigung, und um damit auch evolutionäre Fragen rund um die Entwicklung von Pflanzen-Ameisen-Pilz Mutualismen zu beantworten, sind weitere Untersuchungen mit verschiedenen Pflanzen-Ameisen-Pilz Lebensgemeinschaften wünschenswert.

Supplement

S. 1 All 37 sequenced samples with given sample numbers, full sample name and ant associate (*Azteca alfari, A. constructor, A. xanthochroa).* Affiliations to OTU and genotype is given. Used forward and reverse primer set for PCR, snPCR and Sanger sequencing are given.

No.	**Sample**	**OTU**	**Genotype**	**Ant sp.**	PCR forw.-rev	Seminested primer	Sequencing primers
1	16CecP4_2EpII	2	chaeD-CR-6 FGCec7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
2	ch16CecP5_EpIII	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
5	16Cec24_EpIII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
6	16Cec25_EpIII const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
9	ch16CecP5_EpIII_1	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
10	16CecP6_EpII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
13	16Cec10_EpI_alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
14	16Cec11_EpIII const	2	chaeD-CR-5 Cec14I7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
15	16Cec11_EcIII_const	2	chaeD-CR-5 Cec14I7	const	V9G - cheaD-R	no seminested	ITS5 - cheaD-R
17	16Cec11_leaf_const	2	chaeD-CR-5 Cec14I7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
18	16Cec6_EpII const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
19	16Cec9_stem alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
20	16Cec9_leaf_alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
21	16Cec7_EcIII_alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
23	16Cec1_EcII_const	2	chaeD-CR-5 Cec14I7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
24	16Cec8_EpI_alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
25	16CecP2_EpIII const	2	chaeD-CR-5 Cec14I7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
27	16Cec1_EpIIb const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
32	16Cec14_EpIII_alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
33	16Cec16_EpIII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
34	16Cec16_EcIII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
35	16Cec17_EpIII alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
38	16Cec20_EpIII const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
39	ch16Cec21 EcIII	1	chaeD-CR-1Cec7c	alfari	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
40	16Cec23_EpII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
42	16Cec27_EpII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - cheaD-R	ITS5 - cheaD-R
46	15Cec42_EpI_II_alf	2	chaeD-CR-4 Cec19al1	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
50	15Cec34i10_Fp xanth	3	chaeD-CR-7	xanth	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
51	15Cec36i11_Fp alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
52	15Cec pool2Fp_alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
53	15CecPool_3Fp alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
54	15Cec29i10_yc	2	chaeD-CR-5 Cec14I7	yc	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
57	15Cec33i5_yc alf	3	chaeD-CR-7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
60	15Cec45_EcII_alf	2	chaeD-CR-5 Cec14I7	alfari	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
62	16Cec13_EcII const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
64	16Cec26_EcII_const	3	chaeD-CR-7	const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R
65	16Cec19 EcIII const	CS[MACP1]		const	V9G - cheaD-R	ITS5 - chaeDITS-R	ITS5 - chaeDITS-R

S. 2 Used Primers

Primer	Sequences	Binds in	Source
V9G	5´ TACGTCCCTGCCCTTTGTA 3´	SSU	De Hoog GS and Gerrits van den Ende (1998)
ITS5	5´GGA AGT AAA AGT CGT AAC AAG G 3´	SSU	White *et al.* (1990)
chaeDITS-R	5´AGTACGTGCTACAAGAGC 3´	ITS2	Nepel *et al.* (2016)
chaeD-R	5´ GCCCTACCGCAGTTCCA 3´	LSU	Nepel *et al.* (2016)