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Mycobiomes of sympatric *Amorphophallus albispathus* (Araceae) and *Camellia sinensis* (Theaceae) – a case study reveals clear tissue preferences and differences in diversity and composition

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Abstract

Multiple biotic and abiotic parameters influence the dynamics of individual fungal species and entire communities. Major drivers for tropical plant endophytes are undoubtedly seasonality, local habitat conditions and biogeography. However, host specialization and tissue preferences also contribute to the structuring of endophytic mycobiomes. To elucidate such specializations and preferences, we sampled two commercially important, unrelated plant species, *Amorphophallus albispathus* and *Camellia sinensis* (tea plant) simultaneously at close proximity. The mycobiomes of different tissue types were assessed with high-throughput amplicon sequencing of the internal transcribed spacer DNA region. Both plants hosted different fungal communities and varied in α- and β-diversity, despite their neighboring occurrence. However, the fungal assemblages of *Amorphophallus* leaflets shared taxa with the mycobiomes of tea leaves, thereby suggesting common driving forces for leaf-inhabiting fungi irrespective of host plant identity. The mycobiome composition and diversity of tea leaves was clearly driven by leaf age. We suggest that the very youngest tea leaves are colonized by stochastic processes, while mycobiomes of old leaves are rather similar as the result of progressive succession. The biodiversity of fungi associated with *A. albispathus* was characterized by a large number of unclassified OTUs (at genus and species level) and by tissue-specific composition.This study is the first cultivation-independent high-throughput assessment of fungal biodiversity of an *Amorphophallus* species, and additionally expands the knowledge base on fungi associated with tea plants.

Keywords *Camellia* · Mycobiome diversity · High-throughput metabarcoding · Host specialization · Tissue preferences of endophytes

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Introduction

Fungi associated with living plants have continuously been the subject of intense investigation due to their biological and functional diversity. They act as parasites, pathogens, decomposers or mutualists, such as mycorrhizae (Stajich et al. 2009). Compared with host–pathogen mechanisms in crops and ornamental plants (Dean et al. 2005; Hane et al. 2007), fungal synecology and biodiversity have been rather poorly addressed in the past. During recent years, the study of fungal endophytes has been subject to increasing public awareness (Peršoh 2015). Among other suggestions, it was hypothesized that healthy plants could provide a shelter for various fungal species that become detrimental on the same or on different host plants at different times, developmental stages, genetic constitutions or under different environmental conditions (Kowalski and Holdenrieder 2009), and that some of these fungi could even threaten human nutrition (Islam et al. 2016). An early study of fungal endophytes closes with the question as to, "whether the presence of endophytes in healthy plant tissue hastens the onset of senescence and thus influences the life span of a plant" (Fisher et al. 1986). Recent studies have also demonstrated the beneficial role of plant-associated mycobiomes (Kusari et al. 2013), and approached the evolution and stability of trophic lifestyles among fungal endophytes (Delaye et al. 2013). In this respect, investigations of host and tissue preferences continuously increase our understanding of the general characteristics, diversity, composition and succession of fungal endophytes (e.g., reviewed in Peršoh 2015). Early cultivation experiments have revealed the coexistence of different fungal species in single needles/leaves by biochemical partitioning of resources (Carroll and Petrini 1983). These findings were in agreement with Stone (1988), Johnston et al. (2006), Peršoh (2013) and Zambell and White (2015) who observed partially highly localized, asymptomatic fungal infections in different plants.

However, most of the current knowledge on host and tissue preferences is based on cultivation studies (Petrini and Fisher 1990; Viret and Petrini 1994; Collado et al. 1996; Unterseher et al. 2007; Joshee et al. 2009). Culture-independent approaches in general confirm these initial findings, but additionally reveal an unexpected fungal diversity (e.g., Jumpponen and Jones 2009, 2010; Peršoh 2013).

In tropical habitats, in contrast, it is assumed that host preferences of fungal endophytes are less developed (May 1991; Suryanarayanan et al. 2011; Higgins et al. 2011, Chen and Kirschner 2017), and are often overlaid or even masked by climatic, temporal and spatial parameters (Piepenbring et al. 2015; Matulich et al. 2015). However, both cultivation and cultivation-independent experiments have continuously discovered associations between fungal communities (and species) and their tropical host plants (Arnold and Lutzoni 2007; Unterseher et al. 2013; Solis et al. 2016; Doilom et al. 2016 for cultivation approaches; Kembel and Mueller 2014 for high-throughput sequencing).

The aim of this study was to assess mycobiome diversity and composition of two unrelated but sympatric plants, *Amorphophallus albispathus* Hett. (Hetterscheid 1994) and *Camellia sinensis* (L.) Kuntze. Due to the host plants' distinct taxonomic affiliations (Araceae vs. Theaceae) and the different investigated plant tissues, we hypothesized the presence of clearly visible, plant- and tissue-specific signals among the mycobiomes. To the best of our knowledge, this is the first study that has exhaustively investigated the fungal biodiversity of *Amorphophallus* plants. In contrast, due to the commercial aspects of *Camellia* (tea), knowledge about (cultivable) leaf-inhabiting fungi of this plant is available and serves as a comparison for our data of *Camellia*-inhabiting fungi (Agusta et al. 2006; Kirschner et al. 2009; Chen et al. 2012; Fang et al. 2013; Liu et al. 2015).

Materials and methods

Sampling site at the Mushroom Research Centre (MRC)

Field work took place at one site, the Mushroom Research Centre (MRC, Fig. 1) in northern Thailand, in order to minimize confounding effects of local and temporal variation. The MRC is situated at an elevation of ca. 900 m a.s.l. (19°07.200'N, 98°44.044′E) in a former forested coffee plantation, north of the city of Chiang Mai. The mixed rain forest has developed dense understorey vegetation with many lianas. The canopy trees are dominated by *Castanopsis armata*, *Lithocarpus echinops* (both Fagaceae) and *Dipterocarpus* sp. (Dipterocarpaceae).

Studied plants

Amorphophallus albispathus Hett. (Araceae, Alismatales)

The genus *Amorphophallus* contains approx. 170 species with a paleotropical natural distribution in disturbed or secondary tropical lowland forests (Cusimano et al. 2011). The plants regularly produce one comparatively large, stalked composite leaf (Hejinowicz and Barthlott 2005), which constitutes the only aboveground structure apart from the inflorescence (which was not investigated here). During long periods of dormancy, the plants only consist of a subterranean tuber devoid of a root system. The chosen study species occurred abundantly at the study site. *Amorphophallus albispathus* was first described 22 years ago from central Thailand (Hetterscheid 1994). Two recent studies on this species employed high-throughput

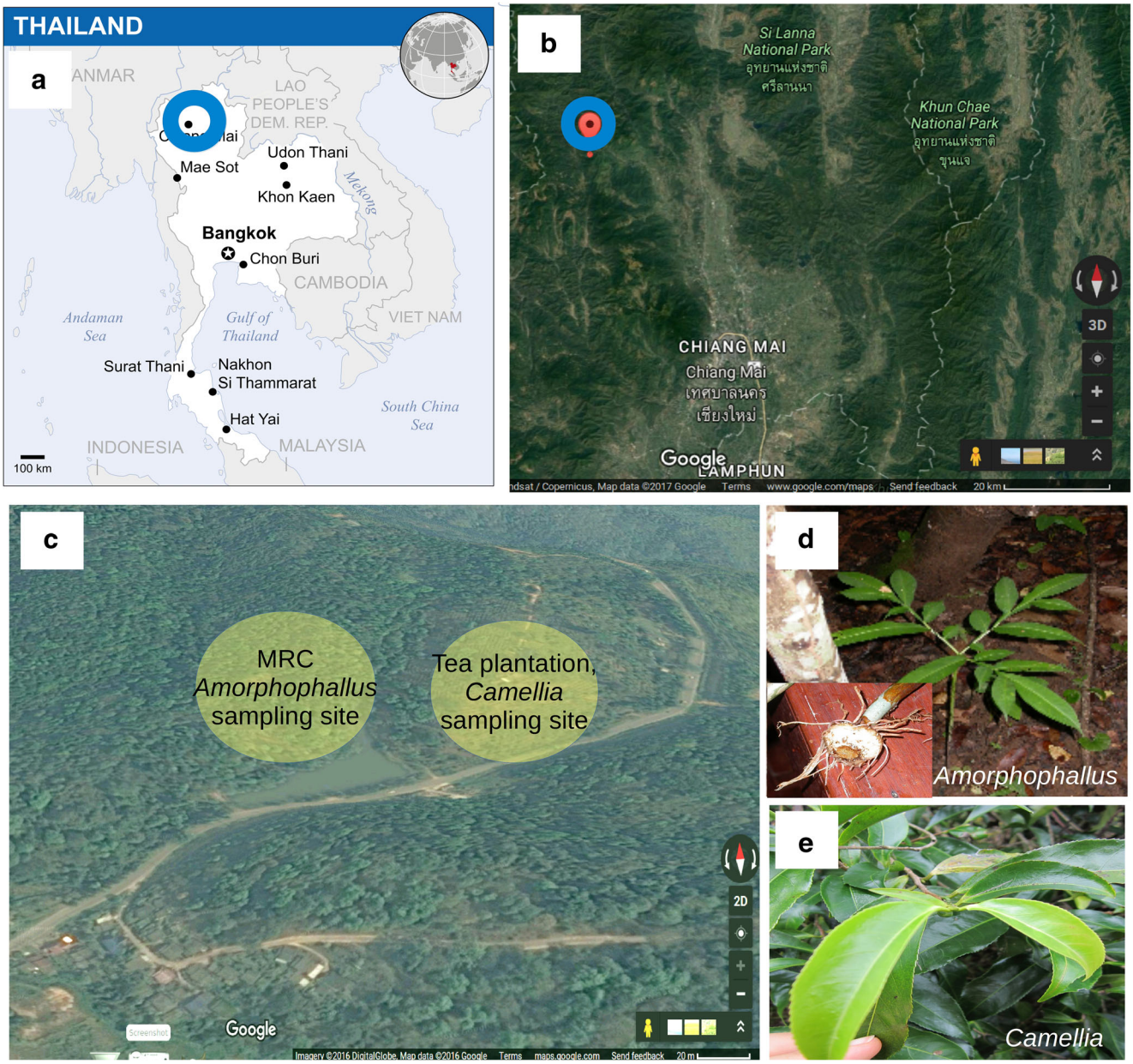

Fig. 1 Characteristics of the study site in northern Thailand at the Mushroom Research Centre (MRC). The *blue circle* in (**a**) shows its location on a map of Thailand (photo source: commons.wikimedia.org, credit: OCHA). **b** A closer satellite view of the area of Chiang Mai with the *blue circle* enclosing the MRC area (photo source and credit: maps.google.com). The aerial view of the sampling site in (**c**) indicate the close proximity of the *Amorphophallus* and *Camellia* sampling site (photo source and credit: maps.google.com). **d** An individual plant of *Amorphophallus* consisting of a single, stalked leaf and a submerged bulb. **e** The upper part of a tea shrub. The topmost, youngest, leaves can be clearly distinguished from the older ones by their *bright green color*

sequencing to identify microsatellites (Zheng et al. 2013) and glucomannan synthesis pathways (Gille et al. 2011).

Camellia sinensis (L.) Kuntze (Theaceae, Ericales)

This species is a perennial, evergreen woody shrub reaching an age of approx. 100 years. Commonly, only the youngest leaves are used for tea production, but some teas (e.g., bricked black tea) are also made from older leaves and twigs (Zheng et al. 2015). The tea produced from leaves of *C. sinensis* belongs to one of the most consumed beverage globally (e.g., Cabrera et al. 2006). Therefore, this plant species is one of the best studied plants worldwide, especially regarding the multitude of antibacterial, antiviral, antitoxic, antifungal, neuroprotective, anti-oxidant, antihypertensive and otherwise bioactive compounds such as polyphenols, catechins and alkaloids

found in the plant (reviewed, e.g., in Wisemann et al. 1997; Harbowy et al. 1997; Friedman 2007). Further studies have focused on the detection of metabolites (Daglia et al. 2014), potentially cancer protective effects of tea products (Cabrera et al. 2006; Yang et al. 2011) or fermentation processes during tea production (Zheng et al. 2015).

Sampling, sample preparation and sequencing

Field work was conducted in August 2013 at the MRC and at the tea plantation at ca. 100 m linear distance. Within a 25-m radius, a total of five fully turgescent *Amorphophallus* individuals were found. They were carefully excavated and dissected into leaflets, petioles, tubers and roots. Tubers and roots were cleaned in water, then the tubers were peeled and the inner tissue was cut into pieces of approx. 0.5 cm in size with a clean razor blade. Leaflets, petioles and roots were chopped accordingly, then all tissues were submerged in 70% ethanol for 5 min and air-dried in a clean and draft-free indoor environment. Tissue-specific fragments were divided into triplicates without keeping track of the identity of the individual plants. For *Camellia*, ten plants were randomly chosen in a similar-sized area and ten young leaves (first and second fully expanded leaves) and ten clearly older leaves from lower parts of the shrub were collected per plant. Fragmentation and surface treatment were the same as for *Amorphophallus* leaflets. All samples were instantly dried over silica gel, stored at ambient temperature and transferred to the laboratory (at the University of Greifswald, Germany).

Fragments from two micro-spatulas were used for genomic DNA extraction with the Charge Switch gDNA Plant Kit (Invitrogen). Multiplexing prior to Illumina high-throughput sequencing followed protocols as described in Unterseher et al. (2016) and Eusemann et al. (2016). In brief, a two-step PCR was carried out. First, a PCR (30 cycles) amplifying the full-length fungal ITS region with the primer pair ITS1F-ITS4 and adding a first pair of short sample-specific identifier oligos. Second, a much shorter PCR (5 cycles) added another pair of identifier oligos, the Illumina-specific adapter and sequencing primer regions. Concentration adjustment, pooling and sequencing were achieved as described in Siddique and Unterseher (2016). The raw Illumina reads are provided under the NCBI SRA accession SUB2198867 (release date 05/05/2017).

Sequence analysis and biodiversity assessment

Amplicons were sequenced in pair-end mode; however, initial analyses of read quality conducted with the free software FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/; accessed November 2016) showed overall lower read quality for R2 (reverse reads covering the ITS2 region) than for R1 reads (forward reads covering the ITS1 region). This led to a much lower recovery rate of R2 reads after quality filtering. All analyses were therefore conducted with R1 reads only.

The bioinformatics environment QIIME (Navas-Molina et al. 2013) and additional tools (Bálint et al. 2014; Bengtsson-Palme et al. 2013) were used for read processing as described in Unterseher et al. (2016). In brief, the workflow consisted of stringent quality filtering using phred scores of ≥30 for at least 75% of the read length, demultiplexing, open reference OTU picking at 97% similarity threshold implemented in the "pick_open_reference_otus.py" command of QIIME (He et al. 2015) and automated taxon annotation with the recent version of UNITE's dynamic reference data set (Koljalg et al. 2013; available from https://unite.ut.ee/repository.php, last accessed July 2016). During these steps, rare OTUs with less than 5 reads were removed from the resulting OTU table (for justification of global removal of rare OTUs, see Brown et al. 2015). With conventional spreadsheet work, the data were further curated by removing tentative non-fungal OTUs (those returning 'unassigned' after taxon annotation; see Online Resource 1).

Fungal diversity was assessed with species accumulation curves, Fisher's alpha (a richness index, representing all species in a dataset), Shannon index (considering both richness and abundance) and two Hill numbers from Hill's series of diversity (N1 = exponent of Shannon index, representing "common" species, N2 = inverse Simpson index, representing "abundant" species according to Hill 1973) in combination with statistical tests (e.g., ANOVA of the multivariate generalized linear models).

Nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCO) based on Bray–Curtis distances of square root-transformed read abundances were used to visualize community composition. The distinctiveness of leaf mycobiomes in different subdatasets was tested with a permutational multivariate analysis of variance using distance matrices (PERMANOVA, 999 permutations). For the analysis of taxonomic composition, the results from OTU clustering and automatic taxon assignment refined by manual queries against NCBI and UNITE databases were used. All data were visualized by highlighting all unidentified fungi, the 20 most abundant OTUs were identified to genus level, and all combined remaining taxa by proportional read counts (relative abundances of all OTUs sum up to 1 for each sample).

Analysis of the tea mycobiome continued in more detail. The most abundant OTUs from *Camellia* were selected and OTUs with similar distribution patterns across samples were identified by hierarchical clustering of a Bray–Curtis distance matrix constrained by sample order, using the 'coniss' clustering.

All biodiversity analyses were performed in R v.3.3.1 (available freely on https://www.r-project.org/, last accessed

July 2016), and the corresponding script and all necessary data files are available as Online Resource 2.

Results

Illumina paired-end sequencing resulted in 3,989,197 raw ITS1 and ITS2 reads, respectively. Sequence processing retained 1,060,681 (26.6%) ITS1 and 36,900 (1%) ITS2 reads. Library size of ITS1 reads did not differ between the two host plants ($t = -0.18$, $df = 13.86$, $p = 0.86$) with an average of 21,708 reads for *Amorphophallus* and 22,606 reads for *Camellia* samples (Online Resource 3, Fig. S1). The median library size was 19,324 and ranged from 3876 to 53,059 reads per sample. The largest library size was obtained from *Amorphophallus* petioles followed by young tea leaves. Similar sequencing depth was recovered from the remaining tissues (roots, tuber, leaflets of *Amorphophallus* and old tea leaves; Online Resource 3, Fig. S2). Sequencing effort was not correlated with OTU richness (Online Resource 3, Fig. S3). OTU abundance distributions of the different host- and tissue-specific assemblages partly deviated from each other (Online Resource 3, Fig. S4, S5).

Richness and other diversity measures

Comparative analysis revealed significantly higher fungal richness for *Camellia* than for *Amorphophallus* (Fig. 2a). Moreover, old tea leaves hosted significantly more OTUs than young tea leaves (Fig. 2b). Roots, tuber and petiole tissues had the lowest richness (Fig. 2c), whereas OTU richness of *Amorphophallus* leaflets almost approached that of old tea leaves (Fig. 2d).

Analysis of further diversity indexes confirmed the results from the richness analyses (Table 1; Online Resource 3, Fig. S6). Mycobiome diversity of *Camellia* was significantly higher for all five indexes than that of *Amorphophallus,* and old tea leaves hosted higher diversity than young tea leaves (Table 1). *Amorphophallus* leaflets showed the highest diversity among the four tissues investigated for this plant (Online Resource 3, Fig. S6).

Community analysis and taxonomic composition

Mycobiomes of the two host plants differed significantly from each other as shown by community analysis with non-metric multidimensional scaling (NMDS; Online Resource 3, Fig. S7), principal coordinate analysis and permutational multivariate analysis of variance (PCO and PERMANOVA; Fig. 3a). Samples of the two plants clearly separated along the most important ordination axis 1, whereas within-group variation was mainly displayed along the axis 2, which held approx. 50% less explanatory power than axis 1 (Fig. 3a). Mycobiome composition of *Amorphophallus* leaflet samples displayed similarities with that of tea leaf samples in general. When analyzing mycobiome composition of young and old *Camellia* leaves, a clear separation of their corresponding samples became apparent (Fig. 3b). In addition, samples of young leaves were more broadly dispersed in ordination space, especially along axis 2 compared with the narrower placement of samples from old leaves (Fig. 3c). However, tests for multivariate within-group dispersion (variance in the average distance of group members to the group centroid) failed to display significant differences (ANOVA: $F = 2.45$, $p = 0.188$). The separate analysis of *Amorphophallus* samples revealed the leaflets as the most distantly placed samples in ordination space (Fig. 3b, Online Resource 3, Fig. S7).

Taxonomic assessment revealed 101 different genera from all major terrestrial phyla of the kingdom Fungi. The proportion of OTUs that could not be annotated to genus level ("unidentified") was highest for fungi associated with tubers of *Amorphophallus* and generally remained high with an average of 52% across all samples (Online Resource 3, Fig. S8). The identified genera (Fig. 4) displayed clear compositional patterns: the four tissues of *Amorphophallus* were highly variable in taxonomic composition, with, for example, clear signs of arbuscular mycorrhiza in roots (e.g., *Claroideoglomus* in sample s02_Root; Fig. 4). The *Amorphophallus* leaflets displayed partly overlapping taxonomic composition with the tea leaves. Taxonomic overlap was the largest between young and old tea leaves, but taxonomic composition of young tea leaves showed higher taxonomic heterogeneity than old tea leaves with one to a few highly dominant taxa per sample (Fig. 4). Old leaves contained several equally abundant genera with a significantly higher evenness compared to young leaves (measured as Simpson index; t test: $t = -3.5$, $df = 9.44$, $p = 0.006$).

The shared mycobiome between old and young leaves is also reflected in the 20 most abundant OTUs (Fig. 5). However, a clear bipartition of young and old leaves is apparent, both in the topology and in the heatmap itself. Samples from young leaves were characterized by individual sets of a limited number of highly abundant OTUs. There was little overlap between the samples, which resulted in long branches within the cluster topology. In contrast, old leaves displayed a more balanced abundance distribution among their corresponding samples.

Discussion

First assessment of the *Amorphophallus* mycobiome

According to our knowledge, this is the first comprehensive published dataset of asymptomatic fungi associated with the genus *Amorphophallus* including all major parts of the plant, except for the inflorescence (for pathogenic fungi, refer to

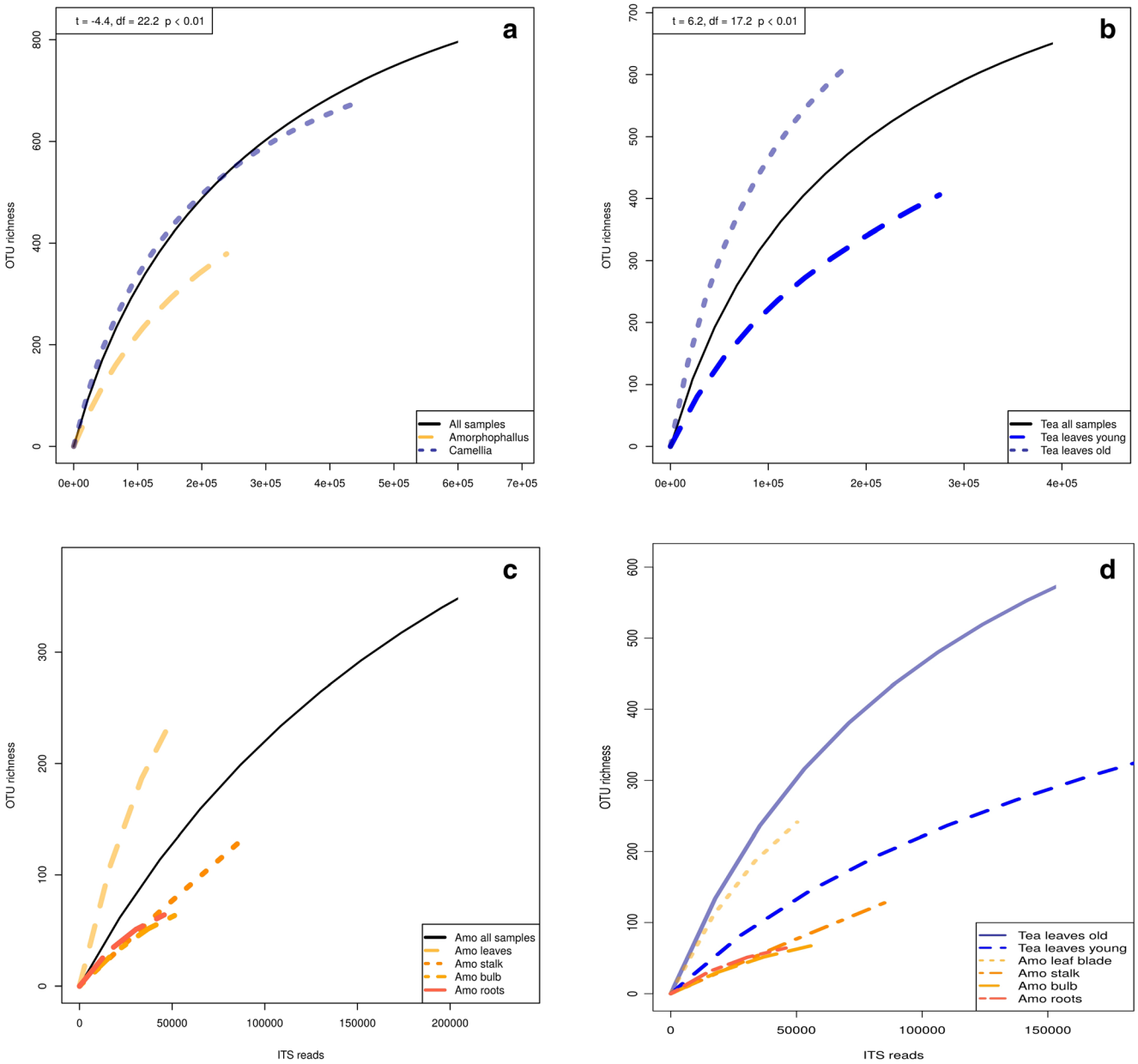

Fig. 2 Comparative analysis of OTU richness obtained from the two host plants (**a**), young and old tea leaves (**b**), the four *Amorphophallus* tissues (**c**) and all tissue samples (**d**)

Misra et al. 2003; Deng et al. 2011; Yu et al. 2014). This plant genus is popular among amateur and professional gardeners due to its unique life style and eye-catching appearance (Beath 1996; Kite and Hetterscheid 1997; Barthlott et al. 2009; Punekar and Kumaran 2010). It is also of commercial interest in the food and pharmaceutical industries (Chua et al. 2010; Zheng et al. 2013) due to the unique biochemistry of the storage organs (Khan et al. 2008; Chua et al. 2010). Our contribution will help to generate a fundamental knowledge base on fungal groups within this plant. The associated molecular data might be compared with the ever-growing curated fungal barcoding database (e.g., plant pathogens: Nilsson et al. 2014; indoor fungi: Abarenkov et al. 2016) in the near future. This might also facilitate the assessment of biological risks (i.e. fungal pathogen load, invasive species) associated with global trade of such plants (cf. Beenken and Senn-Irlet 2016 for invasive fungi of Switzerland).

Leaflets hosted the most distinct fungal assemblage, with significantly lower heterogeneity between samples of the same tissue than between samples of different tissues. Roots, tubers and petioles displayed more similar, but still significantly different, fungal assemblages (Figs. 3, 4). This general pattern suggests a pronounced influence of tissue type (anatomy, morphology, physiology, biochemistry) and small-scale environmental conditions on the detectable fungal composition. Roots are only temporarily present during flowering, leaf

Table 1 Results from diversity analysis using 5 indexes

	Fisher				Shannon				Hill N1				Hill N2				Hill N3			
	Mean value	GLM test for significant difference			Mean value	GLM test for significant difference			Mean value	GLM test for significant difference			Mean value	GLM test for significant difference			Mean value	GLM test for significant difference		
		Sum of squares	SD	*p*		Sum of squares	SD	*p*		Sum of squares	SD	*p*		Sum of squares	SD	*p*		Sum of squares	SD	*p*
Camellia	17.82	538.79	9.96	**0.002**	3.01	8.41	3.87	**0.001**	25.72	1888	11.09	**0.002**	17.23	799	11.30	**0.004**	13.73	460	10.47	**0.005**
Amorphophallus	9.11				1.02				9.41				6.62				5.67			
Young tea leaves	12.17	637.47	22.3	**0.001**	2.38	7.95	2.91	**0.001**	11.66	3956	30.41	**0.001**	8.4	1558	25.75	**0.001**	7.19	854	23.15	**0.001**
Old tea leaves	23.46				3.64				39.78				26.06				20.26			

Mean values of each index as well as corresponding statistical tests to determine significant differences are shown. All diversity indexes showed higher values for *Camellia* than for *Amorphophallus* and for old tea leaves compared with young tea leaves. Significant values in bold

formation and assimilation. Clear signs of Glomeromycota were identified exclusively in root samples (41 OTUs, 43.7% of total root sequences; Online Resource 1), suggesting arbuscular mycorrhizal symbiosis for the *Amorphophallus* species under investigation (Smith and Smith 1997; Brundrett 2006). When the leaf of *Amorphophallus* plants ages and dies, the roots are retracted and the plant consists solely of the dormant tuber. This perennial underground storage organ undergoes various changes in physiology and size throughout a typical vegetation cycle of the plant (Chua et al. 2010), which could lead to a pronounced turnover of fungal composition at different times of sampling. The petiole of *Amorphophallus* leaves is structurally unique with respect to its anatomy and durability (Hejnowicz and Barthlott 2005). It can be considered a distinct organ with fundamental importance for the plant, since it elevates the single photosynthetically active leaf up to 3.5 m into the air for up to 18 months (*A. gigas*; Hejnowicz and Barthlott 2005). The fact that both roots and petiole arise directly from the tuber might explain the relatedness of their fungal assemblages. The leaf blade finally interacts most strongly at the plant–atmosphere interface. Its leaflets contain the photosynthetic machinery and have unique physiological and biochemical properties compared with the other parts of the plant. Taking into account the predominantly aerial colonization of leaves by fungi (Rodriguez et al. 2009), the observed confounding effect of host plant identity (different plant species have different mycobiomes) and tissue type (aerial photosynthetic tissues have similar mycobiomes) was logically consistent (Figs. 3, 4).

Only one OTU was identified across all *Amorphophallus* samples (5.7% of all root sequences, 12.7% of all tuber reads, 27.9% of all petiole reads and 1.2% of all leaflet reads; Online Resource 1). This OTU belongs to one of the most abundant OTUs and was assigned with highest similarity to a type-derived sequence of *Malassezia restricta* (CBS 7877, NR_103585.1, UNITE SH176394.07FU), a yeast that can be considered as a systemically occurring endophytic fungus of *A. albispathus*. Whereas species of the genus *Malassezia* are part of the healthy human skin mycobiota, they also comprise well-known human (animal) pathogens (Gaitanis et al. 2012). It is thus abundantly detected in built environments (Pitkaranta et al. 2008), but has also been found as plant-associated fungi (Tondello et al. 2012; Nasanit et al. 2015; Eusemann et al. 2016) or in plant-pathogenic nematodes (Eberlein et al. 2016). The presence of systemic and for the plant potentially beneficial fungi as well as the comparatively large proportion of unidentified OTUs warrants further investigations of *Amorphophallus* mycobiomes.

The mycobiome of tea leaves

It is commonly accepted that the composition of entire phyllosphere mycobiomes and the behavior of single fungal

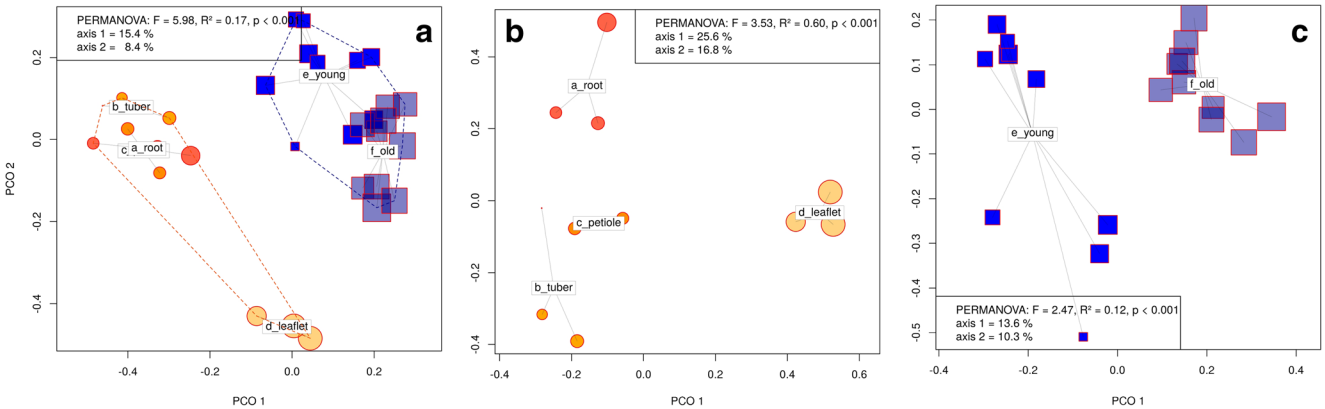

Fig. 3 Mycobiome composition as revealed with principal coordinate analysis (*PCO*) for all data (**a**) and for *Amorphophallus* (**b**) and *Camellia* (**c**) separately

species correlate with physical, biochemical, physiological and seasonal leaf properties (Lodge and Cantrell 1995; Collado et al. 1999; Osono 2008; Olbrich et al. 2010; Jumpponen and Jones 2010; Hunter et al. 2010; Matulich et al. 2015; Unterseher et al. 2016). In turn, these leaf properties are influenced by host species, leaf age, exposure, health status, local climate (season) and microbial colonization (Brossa et al. 2009; Müller and Ruppel 2014; Jensen et al. 2015).

Leaves of *C. sinensis* are rich in catechins, a group of polyphenols known for their antioxidant-related effects (Graham 1992), caffeine, theanine and various other secondary compounds (Song et al. 2012). It has been shown recently that the proportion and quantity of these compounds are dependent on geography and climate (Lee et al. 2010) as well as on leaf age (Lee et al. 2011; Song et al. 2012). Cultivation studies have revealed a clear influence of leaf age and/or seasonality on fungal endophytes of *Camellia japonica* (Osono 2008) and *C. sinensis* (Fang et al. 2013), and additionally demonstrated a "remarkable organizational preference in tea plants" (Fang et al. 2013).

Due to our own project structure, we eliminated seasonal and geographic variation and assessed mycobiome diversity solely in relation to leaf age. All diversity indexes as well as the richness accumulation curves had significantly larger values for old leaves when compared to young leaves. The significant discrimination between old and young tea leaves are retained when analyzing community composition. Principal coordinate analysis (Fig. 3), NMDS (Online Resource 3) and the heatmap-cluster analysis (Fig.

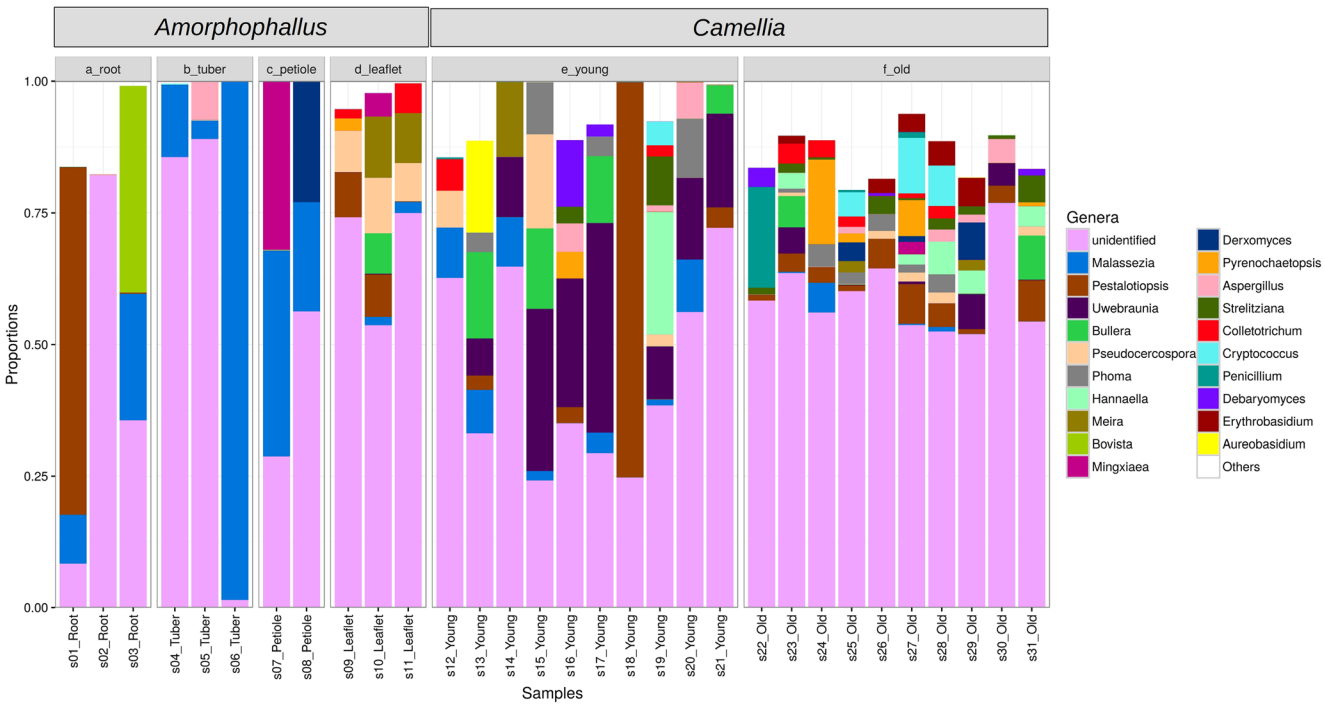

Fig. 4 Taxonomic composition of the 20 most abundant genera displayed as relative read proportions. The remaining 81 genera are subsumed under *Others*. All OTUs which were not identified to genus level are displayed as *unidentified* (the *lowest light-pink-colored bars*)

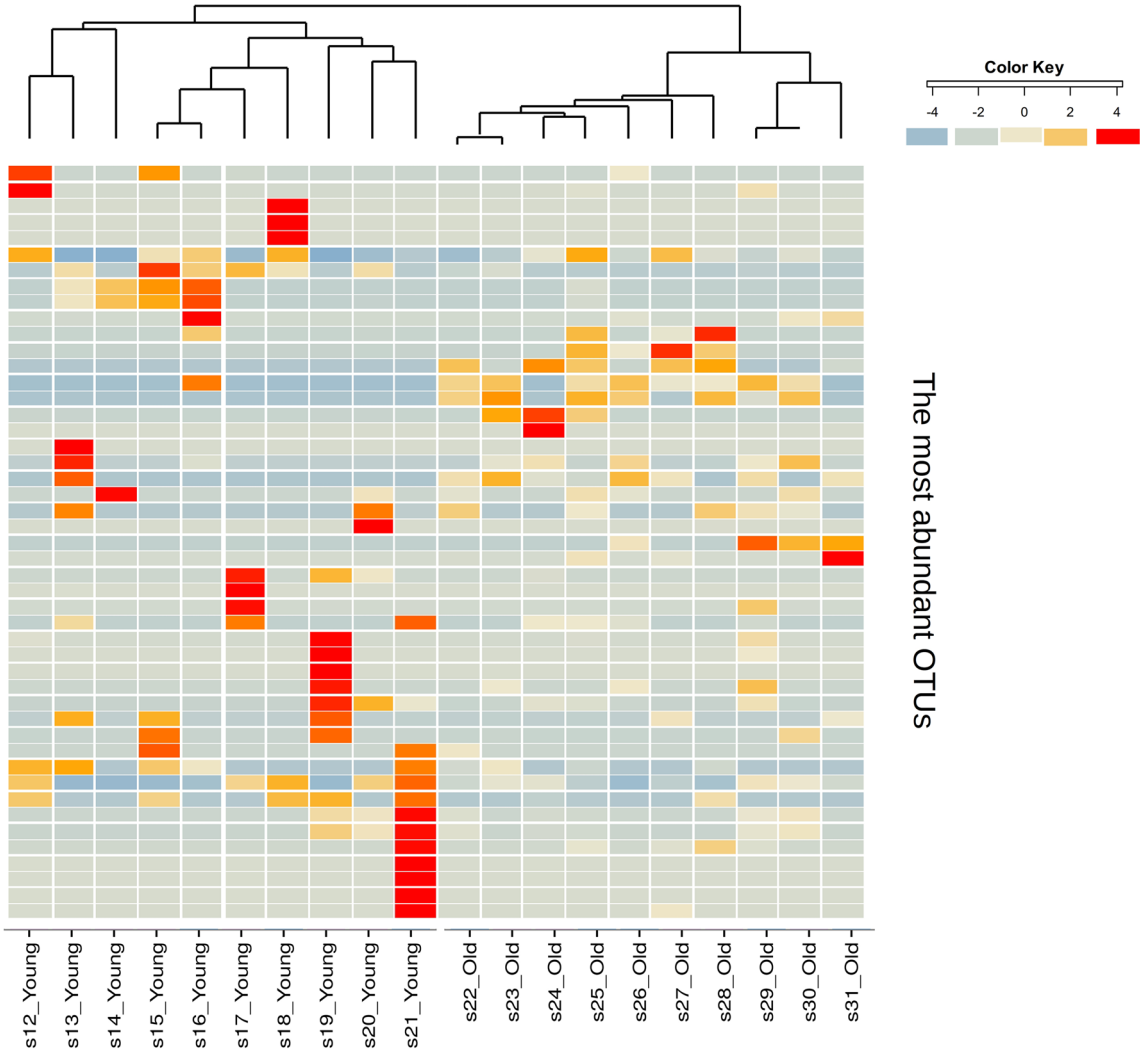

Fig. 5 Heatmap of the most abundant OTUs in the tea leaf mycobiome. The left half displays community composition of young leaves which are characterized by sample-specific patterns of a few abundant OTUs. OTUs from old leaves (*right half*) displays higher levels of co-occurrence and a less pronounced abundance of single OTUs

5) displayed two significantly separated mycobiome groups with a pronounced higher variability of community composition among the young leaf samples. In young leaves, which are plucked for tea production, the proportion of yeast-like taxa was also higher compared with old leaves. Little is known about the role of phyllosphere yeasts of tea plants; however, it is thought that they profoundly influence fermentation processes, reduce the risk of molding and spoilage, and enhance the quality of the end product (Sansone et al. 2007; Xu et al. 2011).

Our own findings for *C. sinensis* generally agree with that of Osono (2008) for *C. japonica*, who identified a positive correlation between leaf endophyte richness of *C. japonica* and age of the leaf measured as years. However, the study of Osono (2008) was to some degree held back by a limited number of samples as well as reliance on fungal cultivation, both of which impose limits on the fungal richness being discovered (see also Langarica-Fuentes et al. 2014 and Siddique et al. 2017 for a comparison of cultivation and high-throughput sequencing).

Currently, we can only speculate about the reasons for the observed high mycobiome variability and dominance of single OTUs in young leaves. Young leaves might be structurally more susceptible to fungal infection and less selective than the older leaves, allowing for an intensified and dynamic competition among the endophytes themselves. In addition the microbial and fungal colonization patterns on leaf surfaces might differ between young and old leaves, thus influencing internally growing fungi in a different way. With increasing age, the leaf chemistry changes: it has been shown that theanine and caffeine concentrations decrease with increasing leaf age, whereas the level of some major catechins (e.g., epigallocatechine) were up to 10-fold higher in older than in younger leaves (Song et al. 2012). Catechins are known for

pronounced bactericidal activity and inhibitory effects against human pathogenic yeasts (Friedman 2007). This will result in selective gradients over time, and our data as well as the work of Osono (2008) suggest that initial stochastic colonization might be replaced by a progressive succession of endophyes, potentially caused by, e.g., leaf chemistry and interfungal competition.

In conclusion, we hypothesize that the generally high diversity, evenness and stable mycobiome of older tea leaves were established due to the specifics of the biochemical profile (and maybe also microbiological composition) of the leaves, which resulted in a continuous selective pressure on the colonizing fungi.

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