Contributions towards a better understanding of sooty mould mycobiomes

DISSERTATION

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Fabienne Maria Anna Flessa
aus *Solothurn, Schweiz*
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Amtierender Dekan: Prof. Dr. Benedikt Westermann

Prüfungsausschuss:

Prof. Dr. Gerhard Rambold (Gutachter)

Prof. Dr. Tillmann Lüders (Gutachter)

Prof. Dr. Heike Feldhaar (Vorsitz)

JProf Dr. Johanna Pausch

Und das Licht scheint in der Finsternis, und die Finsternis hat’s nicht ergriffen.

Johannes 1.5

Im Andenken an meinen Vater Christian Tantscher und meinen Großvater Ernst Tantscher.

Der Besuch der Sammelplätze in 2013 im Reliktregenwald der Mata Atlântica wurde durch Mittel der Deutschen Forschungsgemeinschaft (Projekt DFG RA 731/15-1) unterstützt.

Contents

Zusammenfassung

Als Rußtaupilzvergesellschaftung werden dunkel pigmentierte flächige Mycel-Matten bezeichnet, die sich aus mehreren Pilzarten zusammensetzen und Pflanzenoberflächen eines breiten Spektrums an Wirtspflanzen besiedeln, ohne in die Pflanzengewebe einzudringen.

Die meisten der bisherigen Studien zu Rußtaupilz (SM)-Mykobiomen wurden in den Tropen und Subtropen durchgeführt. Der Vergleich mit Vergesellschaftungen aus temperaten und alpinen Regionen fehlt jedoch. Deshalb wurden in diesem Promotionsprojekt SM-Mykobiome der temperaten und alpinen Region mit denen einer tropischen Region verglichen. Sie werden hauptsächlich von Vertretern der Pilzklasse Dothideomycetes gebildet. In subtropischen und tropischen Regionen existiert zusätzlich ein hoher Anteil an Sordariomycetes. Auf Ordnungsebene überwiegen die Capnodiales, mit einer etwas geringeren relativen Häufigkeit treten regelmäßig auch Pleosporales, Dothideales und Tremellales auf. Auf Familien- und Gattungsebene ist das Spektrum vielfältiger. Die Diversität zwischen Standorten unterscheidet sich signifikant, wenn sich die Standorte in der geographischen Lage, den klimatischen Bedingungen und vor allem in der Wirtspflanzenzusammensetzung unterscheiden. Die SM-Mykobiome der untersuchten Lebensräume setzen sich zum einen aus regelmäßig vorkommenden, charakteristischen Arten bzw. OTUs zusammen, zum anderen aus assoziierten, fakultativen bzw. sporadisch auftretenden Taxa.

In der Literatur wurde eine enge Korrelation zwischen Honigtau und Rußtaupilzauftreten postuliert. Da SM-Mykobiome auch auf Pflanzen ohne diese Nahrungsquelle vorkommen, wurde in diesem Projekt erstmals der Einfluss unterschiedlicher Nahrungsquellen (Auswaschungsprodukte aus Pflanzengeweben, Pflanzensekrete aus Drüsen, Honigtau) auf die pilzliche Diversität untersucht. Stehen nur Auslaugungsstoffe als Nahrungsquelle zur Verfügung, dominieren ubiquitäre Pilze, wogegen pflanzliche Sekretionsprodukte zu spezifischeren Pilzgemeinschaften auf Blättern führen. Die Zusammensetzung der SM-Mykobiome von zwei tropischen Standorten unterscheidet sich signifikant, jedoch mit erheblicher Überlappung zwischen Wirtspflanzen mit Präsenz von Pflanzensaft konsumierenden Insekten (SFI) und solchen ohne SFIs. Auf Blättern immergrüner Pflanzen mit dominierenden ubiquitären Pilzen innerhalb eines Standortes unterscheiden sie sich nicht signifikant zwischen den diesjährigen und letztjährigen Blättern, wenn sich die Nährstoffquelle nicht ändert. Sie differieren jedoch signifikant, wenn sich das Nährstoffangebot mehrjähriger Blätter im Verlauf der Blattalterung verändert. Entgegen der bisher angenommenen Wirtspflanzenunabhängigkeit konnte ein signifikanter Zusammenhang zwischen der taxonomischen Zugehörigkeit auf Gattungs-, Familien- und Ordnungsebene der Wirtspflanze und der Zusammensetzung der SM-Vergesellschaftung festgestellt werden.

Die Sukzession innerhalb einer SM-Vergesellschaftung mit zunehmendem Alter des Blattes wurde vor diesem Projekt noch nicht untersucht. Durch eine Studie an Wirtspflanzen mit annuellen und solchen mit immergrünen Blättern, die während der Vegetationszeit denselben Bedingungen ausgesetzt waren, wobei die immergrüne Gruppe in einem Gewächshaus überwintert wurde, konnten Sukzessionsstadien untersucht werden. Die vorherrschenden Pilze in Erstbesiedlungsgemeinschaften

unterschieden sich in beiden Gruppen. SM Biofilme auf jungen, mehrjährigen Blättern, die durch die Überwinterung im Gewächshaus vom Sporenpool der Pflanzen in offenen Habitaten getrennt waren, unterschieden sich signifikant von denen auf etwa gleich alten Blättern der sommergrünen Pflanzen im Feld. Der Sporenpool im Winter bzw. zu Beginn des Blattaustriebs hat einen wichtigen Einfluss auf die Erstbesiedlung der frischen Blätter. Auch an einer alpinen Wirtspflanze am natürlichen Standort konnte nachgewiesen werden, dass sich die Diversität zwischen unterschiedlich alten Blättern signifikant unterscheidet.

Die relative Menge dunkel pigmentierter Pilze in der 'Kerngemeinschaft' ist an kälteren Standorten deutlich höher als an wärmeren Standorten. Dieses Ergebnis unterstützt zum ersten Mal die Theorie des thermischen Melanismus im SM-Mykobiom. Außerdem weisen epiphytische SM-Pilze einen höheren Pigmentierungsgrad als endophytische Pilze auf derselben Wirtspflanze (*Rhododendron ferrugineum*) auf. Unter den Pilzen des SM-Mykobioms existieren solche, die fakultativ pigmentiert sind und andere, deren Pigmentierung auf verschiedenen Nährmedien nicht variabel ist und deren Hyphen oder Sporen entweder unpigmentiert oder stets pigmentiert sind.

In diesem Projekt wurde zu ersten Mal die Diversität des SM-Mykobioms mit dem endophytischen Mykobiom einer Wirtspflanze verglichen. Beide Mykobiome unterscheiden sich in Bezug auf ihre Zusammensetzung als auch in Bezug auf ihre Beeinflussung durch verschiedene Faktoren wie Blattalter und geographische Lage. Während die SM Biofilme stärker von der Blattalterung betroffen sind und sich signifikant zwischen den Blättern des aktuellen Jahres und denen des Vorjahres unterscheiden, werden die endophytischen Pilze signifikant von der Höhenzone und der geographischen Region beeinflusst.

Die Schlüsselfaktoren, die zum Befall von Pflanzen mit SM Biofilmen in der temperaten Region führen, wurden in diesem Projekt erstmals identifiziert, und es wurde ein Vorhersagemodell entwickelt. Ein längerer Befall mit SFIs ($\geq$ 4 Beobachtungstermine), horizontale Blattstellung und vertiefte Adern haben den stärksten Effekt und führen zu einem 3,7-fach höheren Risiko für das Auftreten von SM-Biofilmen.

Abstract

Sooty mould communities are darkly pigmented planar mycelial mats consisting of several fungal species that colonise surfaces of a variety of host plants without penetrating the tissue.

Most of the research on sooty mould (SM) biomes has been conducted in the tropics and subtropics. However, the comparison with communities from the temperate and alpine regions has been lacking. Therefore, in this PhD project, SM mycobiomes from temperate and alpine regions were compared with those from a tropical region. They are mainly formed by the Dothideomycetes. In subtropical and tropical regions there is also a high proportion of Sordariomycetes. At the order level, the Capnodiales predominate, with Pleosporales, Dothideales and Tremellales also regularly occurring with somewhat lower relative abundance. At family and genus level, the spectrum is much more diverse. Diversity among sites varies significantly when sites also differ in terms of their geographic location, climatic conditions and, above all, in their host plant species. The SM mycobiomes of the investigated habitats are composed of regularly occurring, characteristic species or OTUs as well as of associated, facultative or sporadically occurring taxa.

A close correlation between honeydew and SM incidence has been postulated in the literature. Since SM mycobiomes also occur on plants without this nutritional source, the influence of different nutrition sources (leaching products from plant tissues, plant secretions from glands, and honeydew) on diversity was investigated in this PhD project for the first time. If only leaching substances are available as a nutritional source, ubiquitous fungi predominate, whereas plant secretion products lead to more specific fungi communities on leaves. Composition in SM mycobiomes from two tropical sites differs significantly between host plants with the presence of sap-feeding insect (SFI) and those without, although the groups overlap considerably. On leaves of evergreen plants with ubiquitous dominant fungi within a site, they do not differ significantly between current and previous year's leaves when the nutrient source does not change. However, they differ significantly when the nutritional source changes between young and old perennial leaves during leaf senescence. Contrary to the previously postulated host independence, a significant correlation was found between the taxonomic affiliation at genus, family and order level of the host plant and the composition of the SM community.

Succession within an SM mycobiome with increasing leaf age had not been studied before this project. A study on host plants with annual and perennial leaves exposed to the same conditions during the growing season, but with only the perennial group overwintered in a greenhouse, allowed investigation of succession stages. The predominant fungi in the initial colonisation communities differed in both groups. SM biofilms on host plants with ubiquitous fungi, separated from the spore pool of plants in the field during winter by hibernation in the glasshouse, differed significantly in the composition of SM biofilms on young perennial leaves from the approximately equally old leaves of deciduous plants in the field. The spore pool in winter or at the beginning of leaf sprouting has an important influence on the initial colonisation of fresh leaves. It was also observed on an alpine plant species in its natural habitat that the diversity differs significantly between leaves of different ages.

The relative amount of darkly pigmented fungi in the core SM mycobiome is considerably higher in colder sites than in warmer locations. This result supports for the first time the theory of thermal melanism in SM mycobiomes. Moreover, epiphytic SM fungi are darkly pigmented in a higher proportion than endophytic fungi of the same host plant (*Rhododendron ferrugineum*). Among the fungi of the SM mycobiome, there are those that are facultatively pigmented, and others whose pigmentation is not variable on different culture media, having either unpigmented or permanently pigmented hyphae or spores.

In this project, the diversity of the SM mycobiome was compared with the endophytic mycobiome of one host plant for the first time. Both mycobiomes differ in terms of composition and how they are affected by various factors such as leaf age and geographic location. While the SM biofilms are more affected by leaf senescence and differ significantly between current year's and those of the previous year, the endophytic fungi are markedly shaped by the altitudinal and the geographical region.

The key factors leading to infestation of plants with SM biofilms in the temperate region were identified for the first time in this project, and a predictive model was developed. Prolonged infestation with SFIs (≥ 4 observation dates), horizontal leaf position and sunken leaf veins have the strongest effect and lead to a 3.7-fold higher risk of SM occurrence.

List of abbreviations

ANL Annual leaf from deciduous host plants

BC Brazil Caatinga

BR Brazil tropical rainforest

CYL Leaves from the current year from evergreen host plants

EA European alpine

EC European colline

HTS High Throughput Sequencing

ITS Internal transcript spacer

MB Metabarcoding

MYA Malt-yeast-agar medium

OTU Operational taxonomic unit

PYL Leaves from the previous year from evergreen host plants

SB Single species barcoding

SFI Sap-feeding insect

SM Sooty mould

Introduction

1.1 Sooty mould mycobiomes: characterisation of the symptomatic and disentangling the different terms in use

When saprotrophic dark pigmented fungi first attracted the interest of researchers, they were assigned to a single species called *Fumago vagans* Pers. (1822). The type specimen referred to as '*Fumago*' consists of two completely discordant fungal taxa and the genus name *Fumago* was found to be invalid (Friend, 1965). When dark pigmented epifoliar fungi began to be investigated in more detail, it became clear that the sooty mould (SM) symptom could be caused by a variety of saprotrophic, dark pigmented fungi, which were only superficially adherent to the host plant surface. The very large group of phylloplane fungi has been grouped together with the endofoliar fungal community under the term 'phyllosphere fungi' (He et al., 2012; Lindow and Brandl, 2003; Newton et al., 2010; Vorholt, 2012), or foliar fungi when only mycobiomes of leaves are studied (Busby et al., 2016; Nguyen et al., 2017). Another widely used term is 'fungal epiphyte' or better 'epiphytic fungi'. Such fungi are defined as nutritional guilds on surfaces of living plant parts and include saprotrophic, lichenised, and fungus- and plant-parasitic fungi (Gilbert and Reynolds, 2005, 2002) which can often be obligately parasitic (Ariyawansa et al., 2015; Hongsanan et al., 2014; Wu et al., 2011). The term 'phylloplane fungi' is a general term for fungal organisms that live on the leaves of plants. Various types of fungi are included, from yeasts and yeast-like species to filamentous fungi (Hilber-Bodmer et al., 2017; Koitabashi et al., 2012; Last and Warren, 1972), and its use is similar to the use of the term 'epiphytic fungi' simply indicating localisation on the host plant. These terms are defined broadly and can be applied to dark-pigmented parasitic and non-parasitic communities, as well as communities that are invisible to the naked eye and asymptomatic on a plant surface (the latter are hereafter referred to as epiphyllous non-SM communities).

In general, the term 'sooty mould' is used to describe a dark, dense fungal film generated by a superficial, densely spreading mycelium with a sooty appearance that covers plant organs such as leaves, stems, blossoms and fruits, but does not infect plant tissue (de Filho and Paiva, 2006; Dhami et al., 2013; Faull et al., 2002; Hughes, 1976; Kim et al., 2016; Kim, 2016; Mibey, 1997; Reynolds, 1999; Schoulties, 1980). Nevertheless, they are considered non-parasitic plant diseases (Blancard, 2012) due to the negative impact on the host plant and the economic damage caused by infestation. The black coating leads to a reduction in incident light through physical obstruction, as the SM mycelia act as light filters (de Filho and Paiva, 2006; Nelson, 2008), and leads to an increase in leaf temperature (Wood et al., 1988), at least on leaf surfaces covered by SM biofilms (Kim and Kim, 2017), and a reduction in water content (Santos et al., 2013). SM cover has a variety of negative effects on photosynthesis in affected plants (de

Filho and Paiva, 2006; Wood et al., 1988), although there are controversial findings in this regard (Insausti and Ploschuk, 2018). When plants are covered with SM biofilm, they counteract this disadvantage with increased amounts of chlorophyll (Insausti et al., 2015) along with changes in chloroplast morphology (Kim and Kim, 2017). Nevertheless, SM biofilms can reduce respiration and increasing the water costs for each CO_2 molecule fixed during photosynthesis (Blancard, 2012; Insausti and Ploschuk, 2018). In tropical and subtropical crops such as carambola, citrus, durian, durum wheat, guava, mango, and tomato, infestation by SM leads to economic losses through negative post-harvesting effects (Ancisco et al., 2002; Blancard, 2012; Chomnunti et al., 2014; Fernandez and Knox, 2012; Nelson, 2008; Siriphanich, 2011; Swirski et al., 1997a, 1997b; Warren and Sargent, 2011). The SM-establishing fungal orders are clearly distinguished from Asterinales (Dothideomycetes) and Meliolales (Sordariomycetes), which also form black web-like colonies on leaves, but cause damage to host plant tissues by invading cells for nutrition (Ariyawansa et al., 2015; Hongsanan et al., 2014).

While some authors emphasise a clear link of symptomatology with the occurrence of honeydew and leaf-sucking insects, such as aphids and scale insects (Dhami et al., 2013; Kim et al., 2016; Parbery and Brown, 1986; Perez et al., 2009b), others refrain from such a restriction to honeydew occurrence as a nutritional source for SM fungi (Kirk et al., 2010; Schoulties, 1980). A taxonomy-based definition is another approach. In a strict definition, only fungi from seven families are considered SM, i.e. Antennulariellaceae Woron., Capnodiaceae Höhn. ex. Theiss., Chaetothyriacae Hansf. ex. M.E. Barr, Coccodiniaceae Höhn ex. O.E. Erikss., Euantennariaceae S. Hughes & Corlett ex. S. Hughes, Metacapnodiaceae S. Hughes & Corlett and Trichomeriaceae Chomnunti & K.D. Hyde (Chomnunti et al., 2014; Hughes and Seifert, 2012; Hyde et al., 2013; Reynolds, 1998; Winka et al., 1998). As darkly pigmented fungal aggregations with SM-like appearance are usually not caused by a single species, and phylogenetically unrelated fungi are involved in biofilm formation, a purely taxonomic concept seems insufficient. The term ‘sooty mould’ should therefore only be used to refer to fungal species that belong to the families mentioned above. If, on the other hand, the symptom defined by Schoulthies (1980) is addressed, the term ‘sooty mould’ should be supplemented by further terms like ‘symptom’, ‘community’ (Chomnunti et al., 2014; Flessa et al., 2012, 2021b), or ‘mycobiome’, regardless of the geographic region such as the tropics (Kirk et al., 2010) or the nutritional source such as plant exudates or honeydew from sap-feeding insects (SFI) (Parbery and Brown, 1986).

The SM mycobiome can also be regarded as a type of a non-aquatic or, after rain events, temporary micro-aquatic biofilm (Kim and Kim, 2017), according to Characklis (1990), as it is a heterospecific mycelium as a non-uniform accumulation of cells on a substrate, in which a considerable amount of inorganic or abiotic substances are held together by the biotic matrix. Compared to isolated single cells, transport and transfer processes play a greater role (Characklis, 1990).

While there are several studies in the field of SMs that focus on individual species or are interested in phylogenies, studies on the ecology of SM mycobiomes are still rare. While there have been some studies on tropical and subtropical sites, these have been lacking for temperate or alpine regions.

Little was known about succession within an SM biofilm during leaf senescence. While the close relationship between SM and honeydew produced by SFIs was repeatedly mentioned in the literature, there were no studies investigating the relationship between this and other possible nutrient resources with SM biofilm establishment and composition on plants with deciduous leaves in the European temperate zone. SM mycobiomes and endophytic fungal communities (Hirsch and Braun, 1992; Stone et al., 2004) occur less than 1mm apart. Nevertheless, no study comparing the communities of both habitats has been conducted prior to the present project. Although SM biofilms are globally widespread and repeatedly cause economic losses in crops, no model existed to predict the risk of SM occurrence on host plants. This PhD project therefore addresses gaps in knowledge about the general structure of SM biofilms, how the mycobiome composition changes over time, and factors that influence the establishment and composition of these communities.

1.2 Motivation and objectives

The PhD project focused on clarifying the following aspects of SM mycobiomes:

I) **Diversity** of fungal members in different geographic areas with respect to their taxonomic assignment.

II) **Community-shaping factors**, such as host plant identity and traits, climatic factors, altitudinal zones, and types of nutritional sources.

III) **Structural elements** such as core mycobiomes, affiliation to nutritional guilds, in the context of **successional stages** against the background of increasing host tissue senescence.

IV) **Rule of pigmentation** in relation to microhabitat.

V) **Linkage** of the SM mycobiome with the adjacent endophytic fungal community.

VI) **Prediction model** for SM biofilm infestation on host plants based on key factors.

As it would have been beyond the scope of this PhD project to address all questions at all sites, the individual objectives were investigated in exemplary sub-projects, which are outlined below. Cultivation-related and cultivation-independent methods were used; a discussion on these can be found in Chapter 8.1.

Diversity:

The SM mycobiome was analysed in detail on different native (Flessa et al., 2012, 2021b) and non-native plant species in the Central European colline region (Flessa et al., 2012), in the European alpine altitudinal zone on the evergreen shrub *Rhododendron ferrugineum* (Flessa et al., 2021b; Flessa and Rambold, 2013) and on different native host plant species at two tropical sites in Brazil (Flessa et al., 2021b).

Community-shaping factors:

The differences and similarities in core communities and associated fungi and functional guilds (Root, 1967) of SM mycobiomes from different climatic and geographic zones (Central Europe, alpine, tropical) were compared and the relevant factors for mycobiome composition were determined (Flessa et al., 2021b; Flessa and Rambold, 2013). The dependence of site, host plant order or family, (micro-) climate and different nutritional sources, i.e. leaf leachate, honeydew availability or glandular secretions, on the SM mycobiome was analysed at two sites each in Europe and Brazil (Flessa et al., 2021b).

The relationship between SFIs and SM occurrence was studied on a wide range of *Salix* spp. in a European colline region that are regularly infested by aphids (Flessa et al., 2021a, 2021b). A system with evergreen leaves and an alternative nutritional source could be studied on *R. ferrugineum*, which has active leaf glands, but is not an aphid host plant (Flessa and Rambold, 2013).

Structural elements and successional stages:

The relationship between community development and dependence on substrate longevity in different deciduous tree and shrub species with annual leaves (ANL) in the field was compared with leaves of evergreen plants over-wintered in a greenhouse (Flessa et al., 2012). This study was based on an experimental design that included both indigenous and non-indigenous plants related to Central Europe, such as Mediterranean or tropical plants. Thus, a host plant system in Europe was required, characterised by frequent SM occurrence on perennial leaves in natural locations. Such a system was found in the alpine plant *R. ferrugineum* (Flessa and Rambold, 2013).

Rule of pigmentation:

The proportion of pigmented and non-pigmented species among the core communities as a function of climate and affiliation to nutritional guilds in SM biofilms was analysed in two sub-projects on *R. ferrugineum* in the Alps, *Salix* spp. in a European colline site and on different host plants at two sites in Brazil (Flessa et al., 2021b; Flessa and Rambold, 2013).

Link to the adjacent endophytic fungal community:

By comparing the endofoliar fungal community with the species in the SM biofilms on *R. ferrugineum*, the connection of neighbouring fungal communities on leaves of different ages was investigated (Flessa and Rambold, 2013).

Prediction model:

Based on variables that have a significant influence on the development of SM biofilms, a multivariate model was built that allows predictions on the occurrence of SM biofilms on leaves of willows (Flessa et al., 2021a).

In addition to these studies, which form the core of the PhD project, two separate methodological contributions were made, to an efficient phenotypic classification of fungal cultures using digital imagery (Pietrowski et al., 2012), and an R package with applications for SB sequencing and a pipeline for processing RFLP-based data (Flessa et al., 2013).

Synopsis

2 Objective I: Diversity of sooty mould mycobiomes

2.1 Classes and orders of fungi

Any location on earth where plants can grow, SM can potentially grow as well. Although SMs are sometimes considered typically tropical symptoms due to the fungal families involved (Chomnunti et al., 2014; Hughes, 1976; Kirk et al., 2010), SM mycobiomes also occur in the subtropical (Dhami et al., 2013; Perez et al., 2009a) and temperate zones (Dhami et al., 2013; Flessa et al., 2021b, 2012; Flessa and Rambold, 2013).

While most studies on SMs have focused on describing new species, traits and interactions, and effects of biofilm on the host plants, few analyses of mycobiome alpha diversity (Whittaker, 1972) exist (Dhami et al., 2013; Flessa et al., 2021b, 2012; Flessa and Rambold, 2013; Perez et al., 2009a). Most studies on phyllospheric mycobiomes have not distinguished between fungi of the epifoliar and endofoliar compartments and thus consider the fungal community as a whole. Moreover, the focus has mostly been on the total number of different operational taxonomic units (OTUs) and links to phylogenic categories apart from the phylum level have rarely been made (Delhomme et al., 2015; Fort et al., 2016). Nevertheless, it is possible to use these studies to place the results in a global context and to compare

SM mycobiomes with epiphytic mycobiomes of SM- uninfested leaves. i.e. which do not form visible symptoms (Figure 1).

Figure 1 Sampling sites of all studies evaluated in the overview of the global diversity of non-parasitic epifoliar fungi. SM biofilms were analysed if marked with *, all others were obtained from epiphytic or phyllospheric mycobiomes of SM-uninfested leaves. Sampling locations for the present PhD project are in bold font.

Several studies have followed a metabarcoding (MB) approach with sequence-based assignment, i.e. those conducted in Calabria, Italy (Abdelfattah et al., 2015), Ratchasima, Thailand (Izuno et al., 2016), Changbai Mountain, China (Yang et al., 2016) Grimsel, Switzerland and Bayreuth, Germany (Flessa et al., 2021b), Zhanjiang, China (Yao et al., 2019), Guangdong, China (Qian et al., 2020), and North and South Island, New Zealand (Dhami et al., 2013) and some other studies have used a culture-related, species barcoding (SB) approach, i.e. those in Bayreuth, Germany (Flessa et al., 2012), Grimsel and Julier, Switzerland (Flessa and Rambold, 2013), Itabaiana, Brazil (Flessa et al., 2021b) and Mirandela, Portugal (Gomes et al., 2018). Others have followed a culture-dependent, morphology-based assignment, e.g. in Kyoto, Japan (Osono, Takashi 2002), British Columbia, Canada (Osono, 2007a), Texas, USA (Perez et al., 2009b), and Varanasi, India (Kharwar et al., 2010). Due to the different methods applied, only relative abundances can be considered for comparison.

Comparison of epiphyllous non-SM communities and SM biofilms showed that in both groups, Dothideomycetes are the most dominant class, followed by Sordariomycetes, Eurotiomycetes, and Tremellomycetes (Figure 2). In non-SM mycobiomes, Agaricomycetes and Microbotryomycetes are also common. SM biofilms are thus not substantially different from non-SM epiphyllous mycobiomes at the class level. The relative abundances in our studies (Figure 2, SM-P) do not differ from the whole

of published SM biofilm studies (Figure 2, SM) after excluding unclassifiable taxa. Within Ascomycota, the Dothideomycetes are the largest and most diverse groups (Kirk et al., 2010; Lumbsch and Huhndorf, 2007), followed by the Sordariomycetes. In both groups, species cover a wide range of life history forms, including endophytic and plant pathogenic taxa (Hughes, 1976; Maharachchikumbura et al., 2015; Wijayawardene et al., 2014; Zhang et al., 2006). Despite the similarity of the two groups, the relative abundances differ considerably in the various studies, with the proportion of fungi that cannot be assigned at the class level varying widely from <5% to 85%. This could be due to the methodology applied and the time at which the study was conducted, as the size and reliability of the background database contents are crucial for taxonomic assignment.

Despite some differences in the selectivity of the methods used, the predominant classes are represented at comparable relative abundances across all studies on SM biofilms. However, in MB approaches Tremellomycetes and Leotiomycetes are more abundant, while in species barcoding approaches Dothideomycetes and Sordariomycetes have a higher relative abundance. These differences are obviously due to the selectivity of the two approaches. In the SB approach, the culture medium type may exclude certain fungal groups, in particular biotrophic fungi with specific host interactions, and result in an overrepresentation of saprotrophic fungi (Hudson, 1980; Kirk et al., 2010). Furthermore, basidiomycete records in SB approaches are limited (Sun and Guo, 2012), confirming the inconsistencies between the two approaches (Dissanayake et al., 2018), which is also due to the use of suboptimal primers in the MB approach (Toju et al., 2012a).

Comparing the relative abundances of the dominant classes between sites of the temperate, subtropical, and tropical zones, the proportion of Dothideomycetes is highest in the temperate regions, while that of Sordariomycetes is substantially higher in the subtropical and tropical regions. In the present project, relative abundance of Dothideomycetes was highest in the European alpine site, followed by the European colline site, Caatinga and a relict rainforest in Brazil. Sordariomycetes dominate in the Brazilian sites compared to the two European sites.

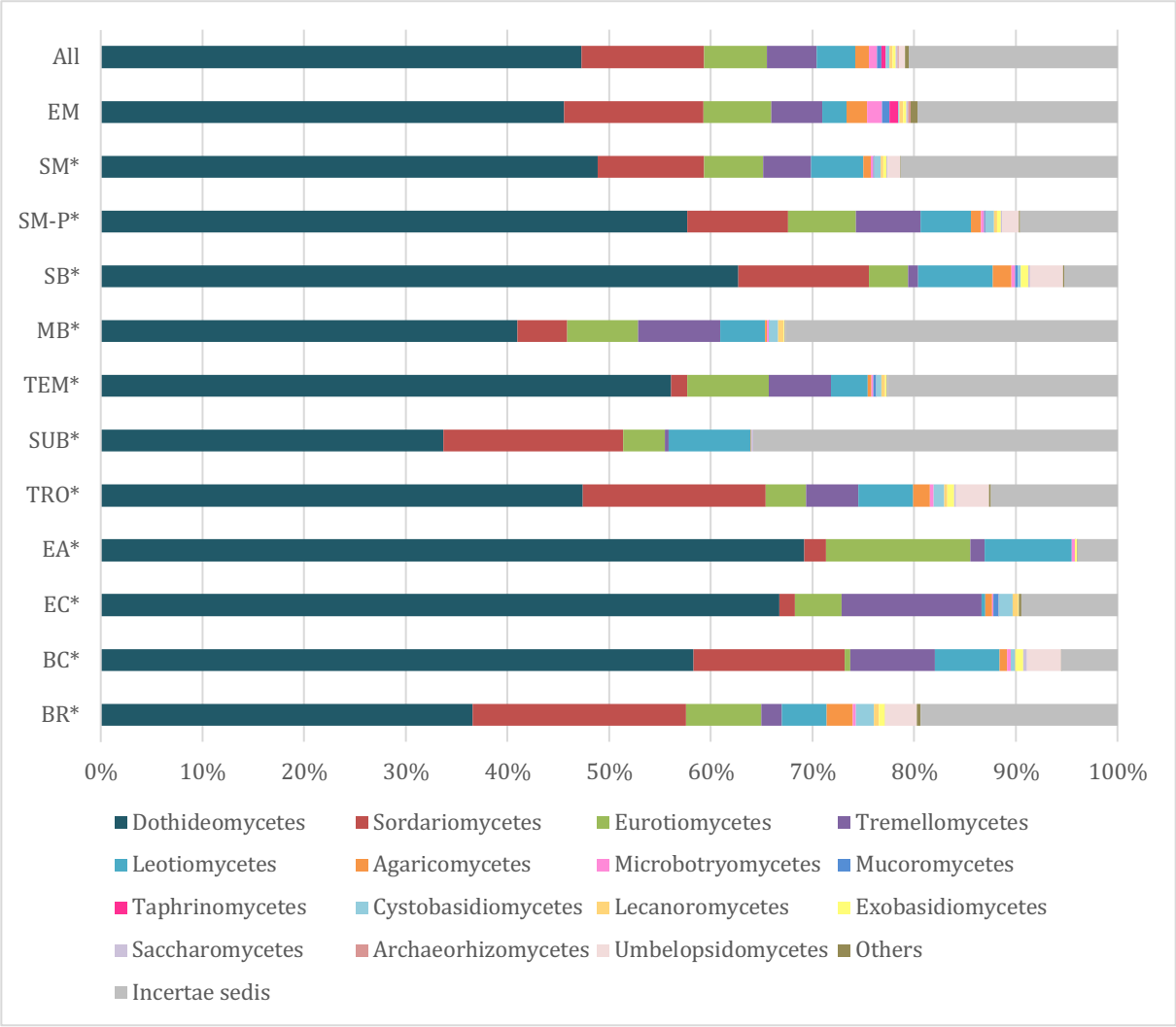

Figure 2 Overall fungal diversity at class level from the studies of the present project compared to the results of other SM mycobiomes and non-SM mycobiomes studies. Fungal classes are listed in the legend on their relative abundance. All classes with a relative abundance of <0.1% are grouped under 'Others', while those marked with * refer to SM mycobiome studies only. All: Relative abundance across all compared sites of studies utilized; EM: Epiphytic mycobiome on SM-uninfested leaves (number of studies/approaches N = 10); SM: Fungal communities on leaves with SM biofilm (N = 5); SM-P: Fungal SM mycobiomes from the study sites of the present project, analysed with a species barcoding (SB) and metabarcoding approach (MB) (N = 6); SB: only SB approach: (N = 3); MB: MB approach only (N = 2); TEM: Epifoliar fungal communities from the temperate climatic zone (N = 5); SUB: Epifoliar fungal communities in studies from the subtropical zone (N = 2); TRO: Epifoliar fungal communities in studies from the tropical zone (N = 2); EA: SM European alpine site, (N = 2); EC: European colline site (N = 2); BC: Brazilian Caatinga site and BR: Brazilian rainforest site (N = 2). BC, BR, EC, and EA are SM mycobiomes from studies in the present project.

Twelve studies were available for comparison of orders, as only classes were examined in three of the abovementioned studies (Dhami et al., 2013; Qian et al., 2020; Yang et al., 2016).

Although the same orders dominate in mycobiomes of both, SM-infested leaves and SM-uninfested leaves (Figure 3), namely Capnodiales, Pleosporales, Dothideales, Hypocreales, and Tremellales, the proportion of Capnodiales is almost twice as high in the SM mycobiomes. The proportion of

Tremellales, Chaetothyriales, and Amphispheriales is higher in the MB approach, whereas the relative proportions of Pleosporales, Dothideales, and Hypocreales are considerably higher in the SB approach. At the Brazilian sites, Hypocreales dominate somewhat more, while Dothideales do so in European sites. Xylariales are only found in the tropical sites. However, the results from the subtropics should be interpreted with caution, as they are based on a single SB study with only morphological assignment.

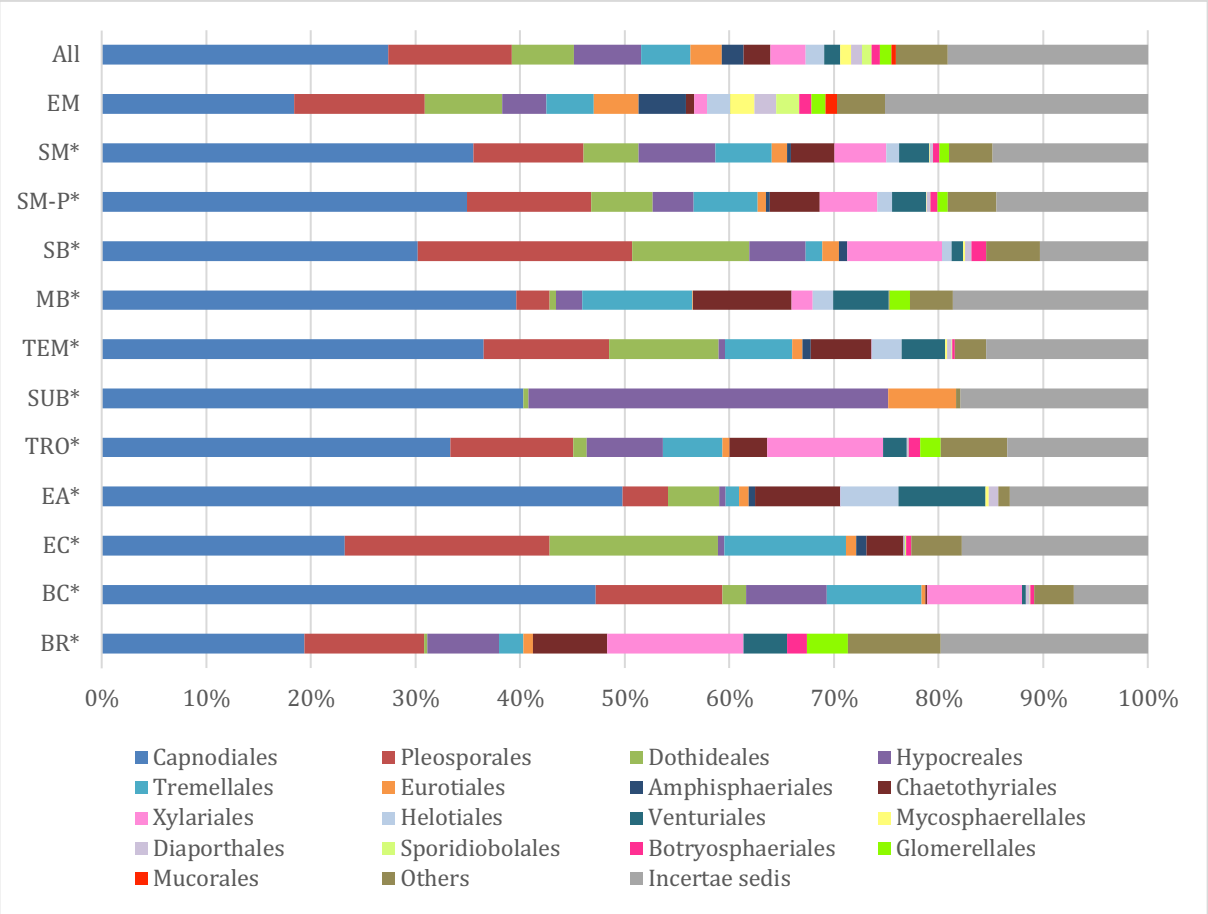

Figure 3 Overall fungal diversity at order level from the studies of the present project in comparison with the results of SM mycobiomes and non-SM mycobiomes studies. Fungal orders are listed in the legend due to their relative abundance. All classes with a relative abundance of <0.5% are grouped under 'Others', while those marked with * only refer to SM mycobiome studies. All: Relative abundance over all compared locations of utilized studies; EM: Epiphytic mycobiome on SM-uninfested (number of studies/approaches N = 8); SM: Fungal communities on leaves with SM biofilms (N = 7); SM-P: Fungal SM mycobiomes from the study sites of the present project, analysed with a species barcoding (SB) and metabarcoding (MB) approach (N = 6); SB: only SB approach (N = 3); MB: only MB approach (N = 1); TEM: Epifoliar fungal communities in studies from the temperate climatic zone (N = 3); SUB: Epifoliar fungal communities in studies from the subtropical zone (N = 1); TRO: Epifoliar fungal communities in studies from the tropical zone (N = 2). EA: SM European alpine site, (N = 2); EC: European colline site (N = 2); BC: Brazilian Caatinga site and BR from the Brazilian rainforest site (N = 2). BC, BR, EC, and EA are SM mycobiomes from studies in this PhD project.

The relative frequencies of the orders differ even more than at the class level in the individual studies. Although Capnodiales were observed in the studies of the present project, their relative

abundances vary strongly. Compared to the other orders, it is still the order with the highest relative abundance in the European Alps, the European colline site, the Caatinga and tropical rainforest sites. However, no dependence on climate is apparent, as the relative abundances in the European Alps and the Brazilian Caatinga are twice as high as in Brazilian tropical rainforest and the European colline sites. The Capnodiales families (Kirk et al., 2010; Lumbsch and Huhndorf, 2007) are leaf epiphytic being associated with honeydew, manifesting as a black coat on surfaces of leaves, fruits and twigs (Hughes, 1976). Moreover, they live as saprotrophs and plant pathogens (Aptroot, 2006; Crous et al., 2009). Pleosporales also occur at all four sites, with their relative proportion being highest in SM mycobiomes in European colline (20%), followed by Brazilian Caatinga (12%) and Brazilian tropical rainforest (11%), and lowest in the examined European alpine SM mycobiome (5%). Within the Dothideomycetes, the Pleosporales are the richest order, comprising a quarter of all species in this class (Kirk et al., 2010). A wide range of ecological traits occur in this order. Moreover they live epi- and endophytically, mutualistically as lichens, antagonistically on plants, fungi or insects, as well as saprotrophically on litter (Kruys et al., 2006; Ramesh, 2003).

At the tropical sites, the proportion of Xylariales is comparable to that of Pleosporales. At the European sites, on the other hand, Xylariales do not occur or play a minor role (0.04%). This result is not surprising, as Xylariales exhibit their highest diversity in the tropics (Becker and Stadler, 2021), and have a saprotrophic or plant pathogenic lifestyle (Kirk et al., 2010).

The Hypocreales are more common in SM mycobiomes at tropical sites. Species classed in the order Hypocreales live as parasitic fungi on plants, other fungi or insects and nematodes, or endophytically (Bushley et al., 2013). These fungi are known to switch hosts and habitats (Prasad et al., 2015) and some of them have effective pathogenicity mechanisms (Yu et al., 2012). Although the Dothideales are, according to literature, fungi that live biotrophically, necrotrophically or saprotrophically on different parts of plants, but rarely on leaves (Thambugala et al., 2014), they are abundant in SM biofilms from the European colline site.

2.2 Most common families, genus and species

SM fungi in the narrow sense, i.e. members of the Antennulariaceae, Capnodiaceae, Chaetothyriaceae , Coccodiniaceae, Euantennariaceae, and Metacapnodiaceae, dominate the SM mycobiomes in the tropics (Chomnunti et al., 2014; Hughes, 1976). However, on closer look, the families and genera differ greatly between the different climatic regions. In Europe, the Cladosporiaceae with *Cladosporium* and Saccotheciaceae with *Aureobasidium* were most abundant (Flessa et al., 2012). Species of the genus *Cladosporium* are globally distributed and occur on various host plants or on other fungi as hyperparasites (Heuchert et al., 2005). They are common in soils and other organic matter (Bensch et al., 2012;

Ellis, 1976, 1971), and some of them are also known as invaders of leaves, either as endophytes (Brown et al., 1998; El-Morsy, 2000; Riesen, 1985) or parasites (Schubert, 2005b), or are common in the epiphytic phylloplane fungal communities (de Jager et al., 2001; Islam and Hasin, 2000; Leventin and Dorseys, 2006; Stohr and Dighton, 2004). The genus *Aureobasidium* includes species that are epi- and endophytic, saprotrophic or biotrophic (Nguyen et al., 2016). In the same climatic zone, but at a higher altitude, i.e. in the European alpine zone in the cantons of Graubünden and Bern, Switzerland, the communities were dominated by *Capnocheirides* (Flessa and Rambold, 2013), which is not assigned to a family. In the tropics, i.e. the Brazilian Caatinga and rainforest, the most frequent fungi belonged to the family Capnodiaceae, including the genus *Capnodium,* and Mycosphaerellaceae, i.e. genus *Mycosphaerella*. *Capnodium* has been identified as predominant in SM biofilms on *Citrus* (Perez et al., 2009b), and the Capnodiaceae as predominant in a SM mycobiome from New Zealand (Dhami et al., 2013). *Capnodium* sp. can produce a range of antibiotics that exhibit varying degrees of antibacterial activity (Herath et al., 2012). In comparison, the genera *Alternaria*, *Ascochyta*, *Aureobasidium*, *Cladosporium* and *Epicoccum* were dominant in epiphytic mycobiomes of SM-uninfested leaves (Gomes et al., 2018; Kharwar et al., 2010; Osono, 2002, 2007a, 2008) and also in the SM mycobiomes of European colline host plants (Flessa et al., 2021b, 2012).

The most common fungi present in at least 30% of the samples among the Ascomycota derived from the studies by Flessa et al. 2012, 2020 and Flessa and Rambold 2013 were combined in an iTol tree (Figure 4). Only eight of these fungi belong to the SM fungi in the taxonomy-based definition, namely *Antennariella placitae*, Capnodiaceae sp., *Capnodiales* sp., *Capnodium* sp., *C. rhododendri* and *Phaeoxyphiella phylicae*.

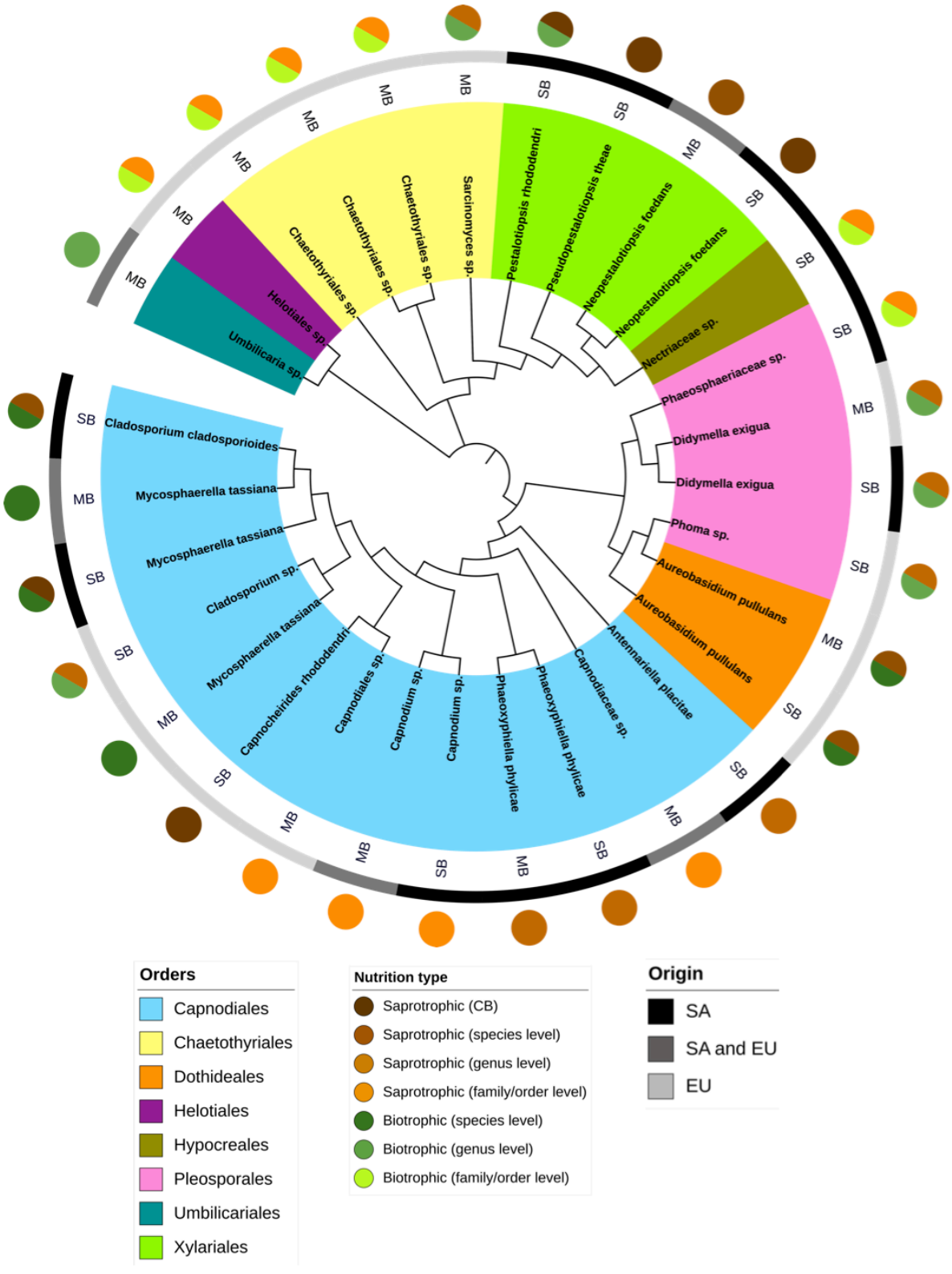

Figure 4 Most abundant OTUs present in at least 30% of the samples among Ascomycota from the SM mycobiomes from the studies of the current project, with taxonomic assignment at species to family levels and with FUNGuild classification at species to family level. The tree was generated using the maximum-likelihood method and Tamura-Nei model (Tamura and Nei, 1993). Analyses were conducted in MegaX (Kumar et al., 2018; Stecher et al., 2020) and visualized using iTOL (interactive Tree of Life) (Letunic and Bork, 2019). Coloured segments indicate fungal orders in the tree. SB: obtained with culture-based approach, MB: detected with metabarcoding; nutrition type is indicated if available in FUNGuild (Nguyen et al., 2016), in the Dictionary of Fungi (Kirk et al., 2010), additional

literature (Maharachchikumbura et al., 2012), or due to cultivability of the respective fungi; origin South America (SA) or Europe (EU).

Conclusions on objective I

The fungal classes of SM mycobiomes do not differ from the fungal classes of epiphytic mycobiomes on SM-uninfested leaves. SM biofilms are mainly formed by the Dothideomycetes. The prevalence is higher in European than at Brazilian locations. In subtropical and tropical regions, the relative abundance of Sordariomycetes is higher than in temperate regions.

At the order level, SM mycobiomes are mainly comprised of Capnodiales. With a slightly lower relative abundance, Pleosporales also regularly belong to the SM biofilms. Xylariales and Hypocreales regularly occur in tropical, while Dothideales are regularly found in SM biofilms of the temperate region.

At the family and genus levels, the spectrum is far more diverse. Predominant genera within the sites of this project are Aureobasidium *(European colline),* Capnodium *(Brazilian Caatinga),* Capnocheirides *(European alpine),* Cladosporium *(European colline), and* Mycosphaerella *(Brazilian rainforest).*

3 Objective II: Community-shaping factors

3.1 Impact of the geographical location and altitudinal levels

The proportion of ubiquitous or shared fungi in the SM mycobiomes at different sites was investigated in two studies of this PhD project and results were summarized using Venn diagrams (Figure 5). Regardless of their continuity or dominance on individual host plants, 37 OTUs were found on both continents (Flessa et al., 2021b). The fungal community composition was significantly different between continents, although there was a high degree of overlap between the two groups ($p = 0.001$, $R = 0.101$). Among the sampling sites European alpine site, European colline site, Brazilian Caatinga and Brazilian tropical rainforest, only 5 OTUs were found in all sites, of which *M. tassiana* was most ubiquitous and dominant. The other four OTUs were not among the predominant at each site. The composition of the fungal community of the four sites differed significantly, and the groups overlapped much less ($p = 0.001$, $R = 0.347$). Sites that are geographically distant from each other and differ in climatic conditions and vegetation composition have SM biofilms whose species composition is significantly different, and the groups are also well separated. Among the four sampling regions in the Swiss Alps from a SB approach (Flessa and Rambold, 2013), five species were present at all sites. Of these, *C. rhododendri*, *Cladosporium* sp. and *Sarcinomyces* sp. were predominant. The four sampling regions Flüelah, Grimsel, Julier and Monstein are similar in terms of host plants and climate. At each site, three altitudinal levels could be clearly distinguished. Across all three altitudinal vegetation zones studied, eight species were

identified that occurred in all zones and were also predominant species. However, according to this vertical zonation, the SM biofilms do not differ significantly in their fungal community composition. Different temperatures did not seem to be relevant for differences in fungal community composition, as Dhami et al (2013) found parallels in community compositions in the subalpine zone with low annual temperatures, and the lowland hills with moderate mean annual temperatures. Temperature and climate alone may not affect the fungal community composition at sites that are rather close (160 km distance) when only OTU spectra are compared.

Species composition is significantly different and the communities overlap less (higher R-value) at sites that do not belong to the same habitat, and are clearly different in terms of climate and vegetation. However, altitudinal gradients or altitude-dependent surrounding vegetation do not lead to significantly different community compositions within a habitat.

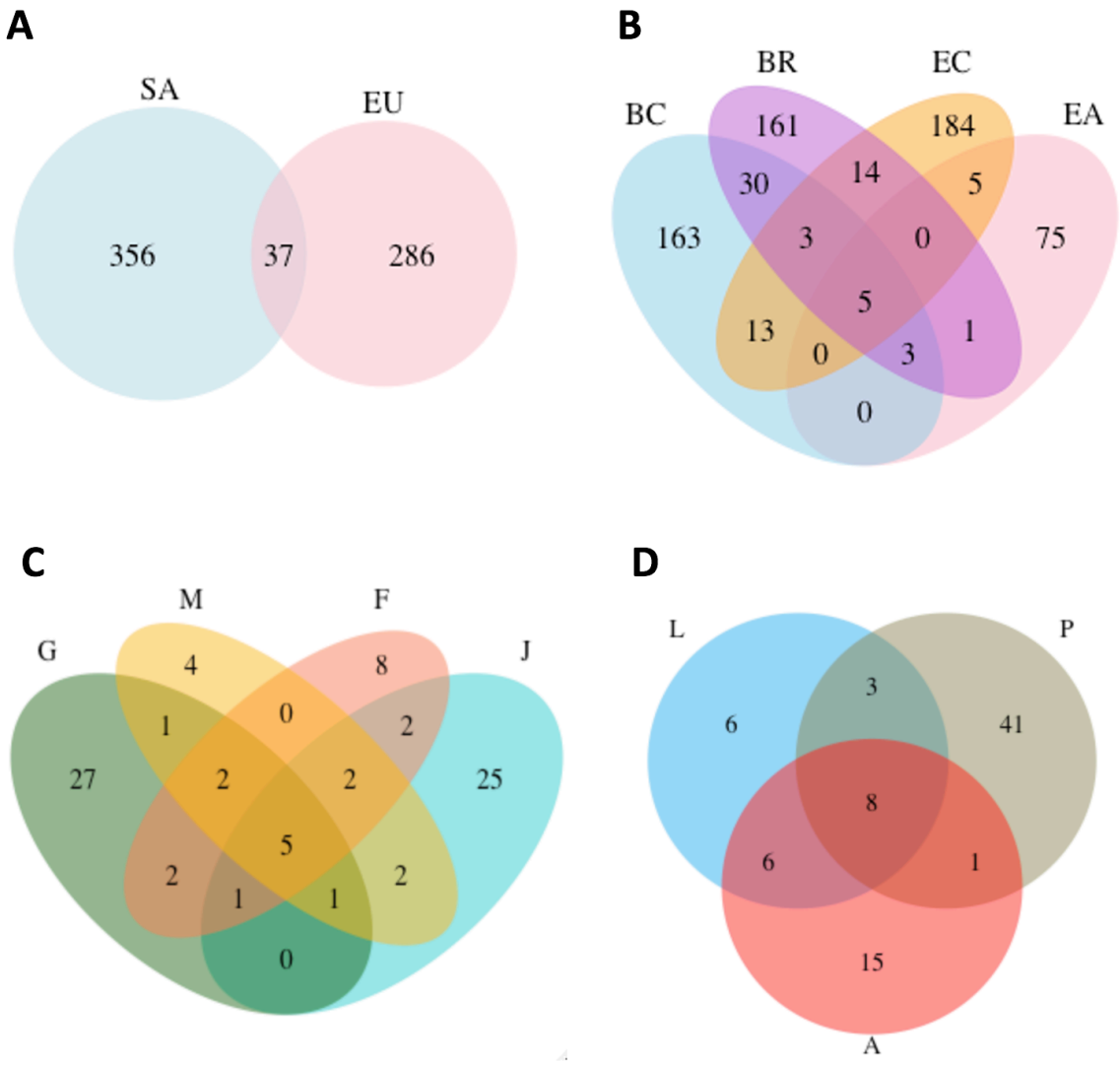

Figure 5 Venn diagrams of shared species in SM mycobiomes between different sites. A and B are based on MB method, C and D are based on SB data. A) Shared OTUs between South America (SA) and Europe (EU), all OTUs from MB from different host plants at two sites in Brazil regarding qualitative biodiversity (pres./abs.) compared with those from Europe (Switzerland and Germany). B) Shared

OTUs between individual sites in SA, i.e. Caatinga (BC) and a rainforest site (BR) in Brazil, and a colline location (EC) and alpine location (EA) in Europe. C) Shared species on *R. ferrugineum* leaves between the sites Flüela (F), Grimsel (G), Julier (J) and Monstein (M) in the Swiss Alps. D) Shared species on *R. ferrugineum* leaves between three altitudinal zones L= European *Larix decidua* forest zone (altitude 1,770–1,980 m), P = mountain *Pinus mugo* zone (altitude 2,000–2,200 m) A = alpine zone (2100–2300 m).

3.2 Host plant selectivity and specificity

Fossil evidence indicates that SMs of the order Capnodiales appeared on the leaves and barks of conifers and angiosperm trees from the tropics to the temperate coastal forests already since the Albian, 100–113 million years ago (Schmidt et al., 2014). SMs are thought to have existed since the existence of angiosperms. The Capnodiales-plant relationship is an example of a well-adapted strategy to successfully colonise a suitable ecological niche (Rikkinen et al., 2003). The SM mycobiome is not restricted to a narrow selection of host plant orders or families. The host plant families from the present project belong primarily to the Magnoliopsida, viz. Anacardiaceae, Apocynaceae, Aquifoliaceae, Asteraceae, Betulaceae, Buxaceae, Celastraceae, Chrysobalanaceae, Cornaceae, Dilleniaceae, Ericaceae, Euphorbiaceae, Fabaceae, Fagaceae, Garryaceae, Malpighiaceae, Malvaceae, Myrtaceae, Peraceae, Rosaceae, Rutaceae, Salicaceae, and Verbenaceae. Flessa et al. (2012) identified three families of Liliopsida, namely Arecaceae, Iridaceae, and Asparagaceae as host plants.

The question to what extent the composition of epiphytic fungal communities depends on the host plant is answered differently by previous studies. There are two approaches dealing with host plant selectivity. One focuses on only one host plant to address specific questions about the influence of different plant parts or leaf age on fungal community composition (Abdelfattah et al., 2015; Arnold and Herre, 2003; Delhomme et al., 2015; Osono, 2002, 2007a, 2008), while the other is concerned with whether SM mycobiome or epiphytic mycobiome of SM-uninfested leaves is host plant-dependent (Dhami et al., 2013; Flessa et al., 2021b, 2012; Fort et al., 2016; Izuno et al., 2016; Kodsueb et al., 2008; Lee and Hyde, 2002; Talley et al., 2002). The dispersal of phylloplane fungal species occurs passively by rain flushing or by other vectors (Andrews and Kinkel, 1986), such as leaf-sucking insects. Members of epifoliar microbial communities may also be transferred by wind dispersal (Brown and Hovmoller, 2002; Bulgarelli et al., 2013; Lindemann et al., 1982; Whipps et al., 2008). This suggests that host plant species plays a minor role if these passive dispersal mechanisms have a strong influence on community composition. Fort et al. (2016) expected that dispersal events between different habitats would homogenize the foliar fungal community composition over growing season periods. However, they showed in a field study that dispersal events are not the major factors shaping fungal communities (Fort et al., 2016). The high similarity in the foliar fungal communities was only found at the beginning of the growing season and diverged towards the end of the season, community richness differed markedly

between host plant species. When comparing the epiphytic mycobiome of SM-uninfested surfaces of mangrove trees, the influence of the month of sampling was a more important factor in determining differences in fungal community composition than the host plant species (Lee and Hyde, 2002). Among the SM mycobiomes, there is evidence that most of them are not host plant specific, but still for some species there are only records on one or a few host plants (Hughes, 1976). The dependency of the host plant taxa was addressed in the present project by investigating the compositions of fungal communities on leaves from a wide range of host plants located in the same geographic area in a SB approach. These included *Acca sellowiana*, *Aucuba japonica*, *Buxus sempervirens*, *Coleonema album*, *Cornus mas*, *Corylus avellana*, *Dietes gradiflora*, *Elaeodendron capense* and *E. orientale*, *Ilex latifolia*, *Lantana camera*, *Quercus robur* and *Q. cerris*, *Tilia cordata*, *Trachycarpus fortunei*, *Salix appendiculata*, *S. atrocinerea*, *S. aurita*, *S. cinerea*, *S. foetidia*, *S. foetidia* × *waldsteinia*, *S. rugulosa*, *S. wallichiana*, *S.* sp. and *Yucca elephantipes* (Flessa et al., 2012). SM mycobiomes did not differ significantly between host plant species ($p = 0.054$, $R = 0.324$). In a study of the effect of honeydew insects, host plant species and geographical location in New Zealand, one of the main findings was that the host plant species did not significantly affect the SM mycobiome (Dhami et al., 2013). It is important to note that host plant dependence has only been tested between plant individuals of the same genus (*Nothofagus*), which is therefore consistent with the results of the present project (Flessa et al., 2012). This changed immediately when tested for dependency on higher taxon levels, i.e. genus ($p = 0.001$, $R = 0.447$), family ($p = 0.001$, $R = 0.414$), and order ($p = 0.001$, $R = 0.441$). Higher taxon levels of the host plant seem to have an influence on the composition of saprotrophic SM mycobiomes compositions in temperate regions. This was also confirmed by using MB data and focussing on host plant family ($p = 0.001$, $R = 0.465$) and order ($p = 0.001$, $R = 0.308$) level from temperate and tropical habitats (Flessa et al., 2021b) and also with a wide range of different host plants, including Anacardiaceae sp., Apocynaceae sp., Asteraceae sp., *Bunchosia* sp., Chrysobalanaceae sp., Dilleniaceae *sp., Dillenia indica,* Euphorbiaceae sp*., Hymenolobium flavum, Hirtella* sp., *Pera bumeliifolia*, *R. ferrugineum*, Sapotaceae sp., *Salix* spp., and *Thyrsodium puberulum*. Even though the atmosphere with its airborne fungal spores is considered a continental and intercontinental corridor for the dispersal of microorganisms including fungi (Barberan et al., 2014; Brown and Hovmoller, 2002; Finlay, 2002; Womack et al., 2010), especially for the most common generalist species such as *A. pullulans*, *Cladosporium* sp. and *Epicoccum nigrum* (Jumpponen and Jones, 2009; Pinto and Gomes, 2016; Zambell and White, 2015), the host plant may have an influence on the actual diversity in SM biofilms on its leaves. It is possible that this depends less on particular taxa than on specific characteristic traits of the host plants. These may be similar in species of the same genus, but more divergent between different genera or higher taxon levels, which is a possible explanation for the contrasting position in the individual studies. Another possibility is that while there is a proportion of ubiquitous fungi, individual species have a stronger host plant affinity. This is at least true for *C. rhododendri* in SM biofilms from European alpine sites, as only one host plant is known for this fungal species.

3.3 Effect of phenotypic traits of host plants

Even if two plants, such as *Tilia* × *euchlora* and *Pyrus calleryana*, have similar leaf morphologies and are exposed to the same environmental conditions, *Tilia* × *euchlora* is regularly infested with SM biofilms but *P. calleryana* is not (Jouraeva et al., 2006). Apart from some similarities, however, the two genera differ in the composition of waxes on the leaf surfaces (Gülz, 1994) and by a much higher proportion of triterpenoids in the genus *Pyrus* (Challice et al., 1980), which apparently influences the development of an SM biofilm. SM biofilms were frequently found on the upper surfaces of leaves (Flessa et al., 2021b, 2012; Insausti et al., 2015; Kim et al., 2016; Santos et al., 2013). This preference for the upper or lower side of the leaf can sometimes be explained by specific host plant structures. The abundance of *Trimmatostroma* sp., which was one of the most common fungi in the study by Lee & Hyde (2002), was higher on the upper leaf surface. This fungus is associated with the salt glands of its host plant *Aegiceras corniculatum* (Goh and Yipp, 1996), which are more abundant on the upper leaf surfaces. The availability and quality of nutritional sources on the leaves also influences the growth location. *Juglans regia* provides two popular nutrition sources for SM biofilms with frequent aphid infestation and subsequent honeydew production and exudates of glandular trichomes (Kim, 2016). The glandular trichomes are found only on the lower side of the leaves, but the SM biofilm only occurred on the upper side of the leaves. The authors assumed that the exudates produced by peltate glandular trichomes alone are not sufficient to support an SM biofilm. However, this is not true in every case. On *R. ferrugineum*, a correlation is found between leaf structure of the host plant and the restriction to the underside of the leaf (Flessa and Rambold, 2013). The upper leaf surface is smooth due to epicuticular wax with a very thick cuticle, but the lower surface has a completely different surface structure with many scale trichomes, e.g. glands (Sosnovsky et al., 2017). SM biofilms are only found on the lower surface, as *C. rhododendri* may be associated with the glands using their secretions as a nutritional source (Flessa and Rambold, 2013). On *Hibiscus rosa-sinensis*, they are also localized on the underside of leaves and are associated with the extrafloral nectaries present there (Ji-Hyun et al., 2015). Other phylloplane fungi with a non-parasitic growth restricted to the underside of leaves have also been detected (Last and Deighton, 1965). The SM mycobiome on *Paulownia* in India has been found on both sides of the leaves, but with *C. cladosporioides* restricted to the lower surfaces (Mehrotra, 1997). However, in the temperate region and in the tropics (Flessa et al., 2021b, 2012), *C. cladosporioides* was also found on the upper surface of leaves. On *Olea europaea*, the SM mycobiome is present on both sides of the leaves, but more frequently on the lower side and mostly in the stoma vicinity (Santos et al., 2013). It is assumed that the gas exchange creates a beneficial microclimate with increased relative humidity in these areas of the leaf.

Among many plant characteristics studied, a horizontal leaf position, deepened leaf veins, rough leaf surfaces and longer leaves had a positive effect on the SM biofilm formation on leaves of *Salix* spp. (Flessa et al., 2021a). However, SM mycobiomes are not restricted to leaves, and are found on all plant structures including twigs, flowers and fruits (Ancisco et al., 2002; Blancard, 2012; Cruywagen et al.,

2015; Fernandez and Knox, 2012; Flessa et al., 2021b, 2012; McAlpine, 1896; Perez et al., 2009a; Serrato-Dìaz et al., 2010; Siriphanich, 2011; Swirski et al., 1997a, 1997b; Warren and Sargent, 2011; Whiteside et al., 1988).

3.4 Influence of nutritional source

According to some definitions, SMs are associated with honeydew of SFIs (Chomnunti et al., 2014; Kirk et al., 2010). However, even if this strict definition is used, SMs are not blind to other nutritional sources available in addition to honeydew. Every plant loses inorganic and/or organic nutrients though rainwater, which leaches these compounds from its leaf tissues. The amount of nutrients provided in that way varies greatly between different host plant species and even leaf ages of the same host plant species (Tomar et al., 2015; Tukey, 1970). SMs are found on plants that are not hosts for SFIs but produce secretions in glands, thus using a third nutritional source. The boundaries between fungi that do not live biotrophically on plants and those that can also invade the host plant under certain circumstances are not that static. SMs are rarely found as single species colonies (Flessa et al., 2021b, 2012; Flessa and Rambold, 2013). If there are other fungi in the community, biotrophism and even parasitism of other fungi are promising nutrition strategies.

Possible nutritional sources for fungi in SM mycobiomes on the leaf surface:

- Leachates: Rainwater dissolves nutrients from tissue of the host plant
- Secretion: Nutrients produced by the glands of the host plants
- Excretion: Insects that feed on the host plant and produce nutrients in the form of honeydew
- Biotrophism: Hostplants or other fungi are used as a nutritional source

3.4.1 Plant leachates: passively available substances from living leaves

Leaching in leaves is a process by which rainwater dissolves organic and inorganic nutrients from the surfaces (Tukey, 1970). The main focus of studies on nutrient fluxes from trees and shrubs has been on effects of the compounds in leachates on the soil microhabitat or on the plant itself, for which the influence of leachates was confirmed (Castells et al., 2005; Stadler et al., 1998). The availability of

nutrients as resources for microorganisms is highly variable on leaf surfaces (Leveau and Lindow, 2001; Monier and Lindow, 2003), which has a direct impact on the fungi that depend on these nutrients. The phenomenon of leaching substances from plants is well studied. As early as 1970, Tukey listed a great diversity of different compounds in his review (Tukey, 1970). The substances leached from plants can be inorganic nutrients, i.e. essential minerals as well as macro- and microelements found in plants. In addition to the inorganic substances, large amounts of organic substances have also been reported, e.g. free sugars, pectic substances, sugar alcohols, all of the amino acids found in plants, many of the organic acids, gibberellins, alkaloids, phenolic substances as well as vitamins. Usually, K, Ca, Mg and Mn as well as carbohydrates are leached out in largest amounts. High quantities of carbohydrates are an ideal basis for fungal growth. For the SM mycobiome, this may be even more important than a honeydew nutritional source, as leaching occurs in virtually every potential host plant. In the studies of this project, SM biofilms were also found on host plants without the presence of SFIs (Flessa et al., 2021b, 2021a).

However, leaching substances are not always beneficial to fungi. Salicylates found in *Salix* spp. and other potential host plants, reduce fungal growth by 50% in higher doses (2.0–5.0 mM, while low doses (0.5 mM or lower) have little or no effect on fungal growth in vitro (Strobel and Porter, 2005). This effect of salicylates was confirmed in an in vivo experiment with *Aspergillus* (Panahirad et al., 2014) in *Pistacia vera* fruits. On the other hand, *Epichloë festucae*, a endophytic fungal symbiont of *Festuca rubra*, expresses a salicylate hydroxylase and is therefore able to bypass this host plant mechanism (Ambrose et al., 2015). Leachates of *Salix* leaves, provoked by heavy rainfalls, can therefore alter the fungal community – not only by providing an additional nutrition source – but also through their antifungal activity. Salicylates in willows are markedly seasonal with a higher concentration in spring at the beginning of the growing season and a significant decrease in June (Förster et al., 2008) in the northern hemisphere. This could be the reason for the observation of SM biofilm occurrence, which only increases strongly towards the end of the growing season (Flessa et al., 2021a). Even if salicylates do not prevent the occurrence towards summer and the end of the growing season, the possible link between late occurrence and SM biofilms community composition needs to be investigated in future studies. Whether *A. pullulans*, which is frequently found in SM biofilms on willows (Flessa et al., 2012), is influenced by salicylates or whether this fungus possesses a salicylate hydrolase also needs to be clarified in further studies.

Antimicrobial compounds such as polyphenolic compounds are also found in host plants of the genus *Rhododendron* (Shrestha et al., 2017). There is a confirmation of the inhibitory effect of extracts from *R. ferrugineum* L. on microorganisms (Lohr et al., 2015). Fungi such as *C. rhododendri* and *Cladosporium* sp. that are very common on *R. ferrugineum* leaves (Flessa and Rambold, 2013) must be able to deal with possible inhibitory substances.

The most persistent fungi in the SM mycobiome at the Caatinga site, where SFI were also found on leaves, were Capnodiales sp., Capnodiaceae sp., *Capnodium* sp. and *M. tassiana* with a presence in over 50% of the samples, while at the tropical rainforest site, where no SFIs were found,

Neopestalotiopsis foedans and *M. tassiana* were among the most persistent fungi found. *M. tassiana*, as a ubiquitous fungus, seems to be able to access many different nutritional sources, while *N. pestalotiopsis* copes better with leaching substances than *Capnodiales* spp.

The predominant fungi in SM mycobiomes on leaves where only leaching products are present are characterised in all studies of this project by fungi that are ubiquitous, i.e. *A. pullulans*, *Cladosporium* sp. and *M. tassiana*. SMs in a taxonomy-based definition, such as *Capnodium* sp. or *C. rhododendri*, do not occur (or are less frequent) in this type of nutritional niche, viz. they are co-dominant rather than predominant.

3.4.2 Secretion: Nutrients actively produced by the plant

SMs can live closely related with plant exudates (Rikkinen et al., 2003; Schmidt et al., 2014), i.e. exudates by extrafloral nectaries, such as on the annual herbaceous plant *Hibiscus cannabinus* L., an economically important fibre plant from the subtropics and tropics (Choi et al., 2015). On *Hibiscus rosa-sinensis*, an evergreen shrub cultivated in the subtropics and tropics, the SM biofilm was observed in the junction between the leaf blade and petiole and dispersed from there along furrows on the abaxial surface (Ji-Hyun et al., 2015). This local occurrence in this specific region is due to the extrafloral nectaries located there. The SM biofilms are dominated by *Leptoxyphium kurandae*. In *R. ferrugineum*, the SM mycobiome was never found on the upper leaf surfaces during both studies of this project (Flessa et al., 2021b; Flessa and Rambold, 2013). A clear preference for one of the leaf surfaces is often associated with an alternative nutritional source to the omnipresent leachates. A *Trimatostroma* species common on *Aegiceras corniculatum* leaves, is associated with the salt glands of its host plant (Goh and Yipp, 1996). As salt glands are more abundant on upper leaf surfaces, it is no surprise that the abundance of the *Trimatostroma* species is higher on this surface (Lee and Hyde, 2002). Leaf glands are common in several plant taxa. In *R. ferrugineum*, a smooth upper leaf surface due to epicuticular wax and a very thick cuticle (Sosnovsky et al., 2017) prevents the host plant from significant nutrient losses due to leaching by rainfall and also deters microorganisms from adhering to the phylloplane. The lower leaf surface has a different texture and appearance as it is rough and has many scale trichomes, e.g. glands (Sosnovsky et al., 2017). On *R. ferrugineum*, SM biofilms were found on twigs or on the lower leaf surface, but never on the upper leaf surface. An SM biofilm on the lower leaf surface is unusual among SM mycobiomes and the most obvious explanation for that non-standard location is the asymmetric arrangement of the foliar glands of its host. The production of gland secretions can only be observed in current year leaves (CYL) (Flessa and Rambold, 2013). In older leaves, i.e. leaves of the previous year (PYL), this nutrition source dried up. *C. rhododendri* may be associated with the glands using their secretes as a nutrition source (Flessa and Rambold, 2013) and is therefore more abundant than any other

fungal species during the time of leaf gland activity. Once that nutrition sourced dries up, as can be observed in PYL, the advantage of *C. rhododendri* over other fungal species is immediately be eliminated. The place of most frequent fungi is now taken by a very common fungal genus on plant surfaces, namely *Cladosporium*. The shift in SM mycobiome composition from CYL to PYL on *R. ferrugineum* is strongly related to its leaf gland activity and the resulting change in nutritional source between leaf generations.

3.4.3 Excretion: honeydew produced by leaf sucking insects

Honeydew is the excretion product of SFIs on plants (Wari et al., 2021). Its deposition on leaves leads to a reduction of CO_2-assimilation and increases leaf senescence (Dik et al., 1991; Rabbinge et al., 1981) and is therefore an additional disadvantage for host plants parasitized by SFIs. It is produced by insects in the order Hemiptera, suborder Homoptera, such as whiteflies, aphids, soft scale insects, leafhoppers, psyllids and mealy bugs (Barr, 1987; Drees and Jackmann, 1998; Kwee, 1988; Nandi et al., 2020). It is an excellent nutritional source for the microbial community on leaves as it consists of various sugars and some amino acids in the form of an aqueous solution (Auclair, 1963; Maurizio, 1985; Way, 1963). It contains, for example, fructomaltose (Gray and Fraenkel, 1953), stigmatriose, stigmatetrose, and stigmapentose (Bogo, 2003). Its composition and amount vary significantly between aphid species and even within one species it depends on the age of the insects (Fischer et al., 2002; Hendrix et al., 1992; Hertel and Kunkel, 1977; Völkl et al., 1999).

The effect of SFI occurrence on the development of an SM biofilm was investigated for the purposes of this project during a field study on different willow species. It was confirmed for the first time that SFIs also have a significant positive effect on SM biofilm development in plants with annual leaves of the temperate region, which had previously only been experimentally verified in a evergreen host plant in the tropics (Shukla et al., 2017). Despite the large temporal gap between the main SFI season and the main SM biofilm occurrence, a significantly higher occurrence of SM biofilms was found on host plants with SFI at four or more observation dates (Flessa et al., 2021a).

SFIs alter throughfall solutions in affected trees (Stadler et al., 2006; Stadler and Michalzik, 1998). In a study on the effect of aphids on nutrient fluxes and thus on microorganisms living on needles of Norway spruce, significantly more bacteria, yeasts and filamentous fungi were found in the period when aphids were also most abundant (Stadler et al., 1998). In the month before, when aphid abundance was low, microbial abundance was low as well. The epifoliar fungi were still more abundant on previously aphid-infested twigs in September than on the control twigs without aphids, which is consistent with the results of the study on willows in this project.

The composition of the fungal community differs significantly, but with a large overlap between host plants with and without SFI occurrence (Flessa et al., 2021b). The main consumers of honeydew among the fungal community are saprotrophic (Dik et al., 1991). Dhami et al. (2013) investigated the influence of two scale insects, viz. *Coelostomidia wairoensis* and *Ultracoelostoma brittini*, on the SM mycobiome. Due to the study design with one scale insect being associated with a single host tree species (Dhami et al., 2013), it has not been possible to say with certainty whether the host plant species or the scale insect species is the main factor for the observed differences in SM biofilm composition. In the present project, the SM mycobiome from the tropical Caatinga site with SFI occurrence differed significantly from that of the tropical rainforest site without SFI occurrence (Flessa et al., 2021b). Capnodiales sp., Capnodiaceae sp., *Capnodium* sp., which were the most persistently occurring fungal OTUs among all samples from the Caatinga site, could thus benefit more from honeydew than other, more generalist fungi. However, this result cannot be transferred to the temperate colline sites, where mainly ubiquitous fungi (*A. pullulans*, *C. cladosporioides*, *M. tassiana*) predominate in the SM mycobiome (Flessa et al., 2021b, 2012).

3.4.4 Biotrophism

According to the guild assignment via FUNGuild database (Nguyen et al., 2016), most of the fungal species in the study of the SM mycobiome on leaves in the temperate colline zone (Bayreuth, Germany) are mixotrophic and even purely biotrophic, i.e. pathotrophic and symbiotrophic (Figure 6). However, these results should not be over-weighted, as all fungi in this study were obtained by a SB approach, with which strict biotrophs are not found. Nevertheless, due to their cultivability, they must also be able to feed non-biotrophically. In the SM mycobiome and endophytic fungal community on the alpine plant *R. ferrugineum*, the proportion of exclusively saprotrophic fungi decreased from the younger to the older leaves, while in both groups in PYL the proportion of mixotrophic fungi increased and exclusively pathotrophic fungi were also found. Again, the results are critical, as this fungal community composition was analysed using a SB approach. At both Brazilian sites, the proportion of strictly saprotrophic and mixotrophic fungi was similar. However, the proportion of exclusively pathotrophic fungi was considerably higher than in the SM biofilms from the temperate or alpine regions. In the tropics, for example, *Pestalotiopsis* spp. were observed (Flessa et al., 2021b). *Pestalotiopsis* sp. is probably capable of penetrating the epidermis (Lee and Hyde, 2002). Although the entries in the FUNGuild database are not comprehensive for every fungus, as fungi are also described as strictly biotrophic but still cultivable (Flessa et al., 2021b), it can be concluded that both saprotrophic and strictly or temporarily biotrophic nutritional types occur in SM mycobiomes of all sites. The SM mycobiome can therefore be described as mixotrophic. However, this should be investigated in further studies, as a single

FUNGuild record cannot be regarded as fully informative, and for many fungal species the versatility of the nutritional strategy may not have been investigated yet.

As individual members of the SM mycobiome are not strictly saprotrophic, it is possible that they additionally or exclusively feed on living material of the host plant or parasitise other fungi within the SM biofilms. Most fungi in the core community analysed in an MB approach are non-pathogenic, while some plant pathogens were found among the associated fungi (Flessa et al., 2021b). In the core community, there is only one OTU that is potentially fungicolous due to its membership to the family Phaeosphaeriaceae, as there are fungal parasites within this family in addition to saprotrophic species and plant parasites. However, this remains highly speculative. Within the associated fungi, which were present in at least 10% of the samples, only one other OTU was found, which could be fungicolous based on its assignment to the genus *Fusarium*, as it is classified under lichen parasites in FUNGuild. Fungicolous fungi thus play a minor role within the SM mycobiomes.

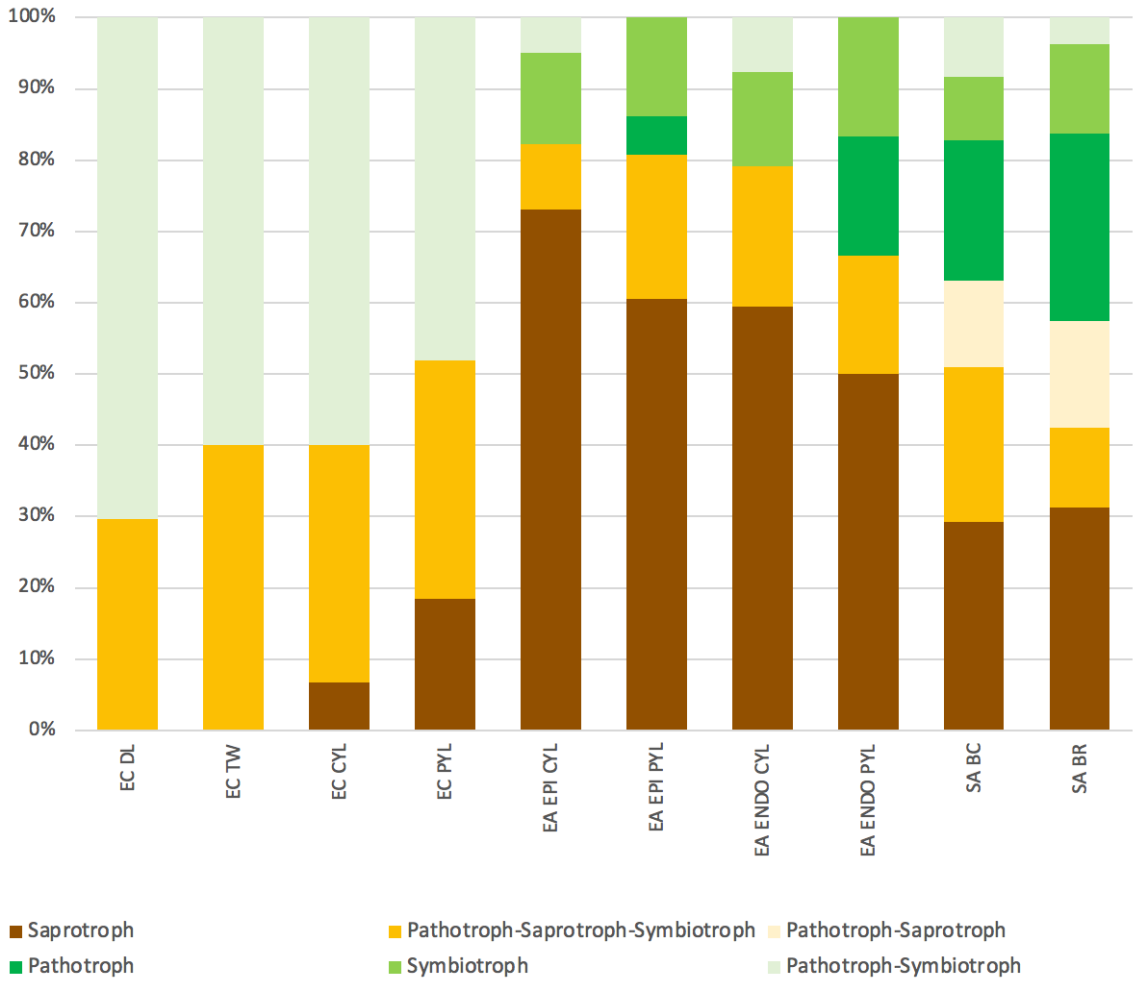

Figure 6 Nutritional type based on assignments via FUNGuild database. EC: data based on the study by Flessa et al. 2012 from Germany, Bayreuth; DL = SM mycobiomes on deciduous leaves; TW = SM mycobiomes on twigs of deciduous plants; CYL = SM mycobiomes on current year's leaves of plants with perennial leaves; PYL = SM mycobiomes on previous year's leaves; EA: data based on the study by Flessa and Rambold (2013) on SM mycobiomes on *R. ferrugineum* in the Alps of Switzerland; EPI = SM mycobiomes; ENDO = endophytic fungi; SA BC and BR: data based on MB approach

samples from the Brazilian sites of Flessa et al. (2021). Data from EC and EA originate from a the SB approach (Flessa et al., 2012; Flessa and Rambold, 2013). These fungi are at least temporarily non-biotrophic.

Conclusions on objective II

SM mycobiome diversity differs more between sites when the sites also differ in terms of their geographic location and host plants. SM biofilms at different elevation levels within similar sites with the same host plants did not differ significantly.

Unlike the epiphytic mycobiomes of SM-uninfested leaves, there is a relationship between the taxonomic affiliation of the host plant and the composition of the SM mycobiome, although this relationship is only significant at the genus, family, and order levels.

Localization of SM biofilms on plants is not random, but is linked to nutrient availability. Nutrients may be available passively through accumulation of leachates or through active production of nutrients by the plant or plant-associated insects on the host plant.

If only leachates are available as a nutrition source, the SM biofilm is predominantly composed of ubiquitous fungi.

The availability of secretion products leads to the dominance of a more specific fungus on R. ferrugineum *than when only leaching products are available.*

Willows with a longer SFI infection period are more likely to have a SM biofilm than plants without or with a shorter SFI presence. Fungal community composition differs significantly between host plants with SFI presence and those without, but the groups overlap considerably.

Although SMs are often described as saprotrophic in the literature, there are some fungi within the SM mycobiome with a mixotrophic or even biotrophic nutritional type. Fungal parasitism is of low relevance.

4 Objective III: Structural elements and succession

4.1 Succession and the point of a steady state in the phylloplane sooty mould biofilms

4.1.1 Successional stages and seasonal variations

Plant surfaces, especially leaves of plants as habitats for fungal communities, are characterized by their transformation processes (Mechaber et al., 1996). Unlike many other habitats, they undergo

major changes during their lifetime. The leaf is predestined by its biology to promote succession and variation in its phyllosphere community. Succession is a non-seasonal, oriented and continuous pattern of populations establishment and decline in an area (Begon et al., 1998). In contrast, seasonality is a periodic process (Fretwell, 1972; Williams et al., 2017) without determined direction. Species alternate in periodic changes of conditions on the same substrate. Seasonal variation in the environment of a fungal community on plant leaves are large. Epifoliar fungal communities are subject to all these changes and each fungal species is either able to cope with these changes or will cease to exist as part of the community in the future. The composition of the fungal community in a current state is like a negative for all the factors currently affecting the niche, and the succession is influenced by ongoing processes during leaf senescence in that niche.

On individuals of the host plant genus *Salix* sp. with ANL, an increase in SM biofilm occurrence could only be detected from July onwards (Flessa et al., 2021a). At the beginning of the vegetation period, they could not be detected on leaves of almost all plants. The maximum was observed towards the end of the growing season before leaf fall. The late occurrence could possibly be related to changes in the substrate, as the concentration of salicylates in willows is highest in March and decreases significantly during the growth season (Förster et al., 2008). Salicylates can have an inhibitory effect on fungi (Strobel and Porter, 2005), but some are also able to overcome this mechanism by producing a salicylate hydrolase (Ambrose et al., 2015). However, when the weather conditions are more favourable for SM biofilms from July onwards, this effect could also be stronger. Since not only the leaf as a niche changes over time, but itself is also exposed to seasonal climatic fluctuations, it is not always clear to which factor the changes in community composition are due. Lee & Hyde (2002) found the same fungal species spectra in the two evergreen host plants *Kandelia candel* and *Aegiceras corniculatum*. However, they observed a host plant-specific shift in fungal abundance, with the highest abundance on *Kandelia candel* occurring between summer and winter, while on *Aegiceras corniculatum*, the highest peak was found in August and decreased in winter. The study period was only one year, no distinction was made between different leaf ages and only SM-uninfested leaves were investigated. Epiphytic mycobiomes from SM-uninfested leaves of the evergreen host plant *Olea europaea* showed significant seasonality (Gomes et al., 2018). Again, no distinction was made between leaf ages, and the observation period was also only one year. In both studies, it cannot be excluded that with a different study design, a successional process would have been observed instead of seasonality. Not every epifoliar fungus is sensitive to seasonality: the epiphytic *Pestalotiopsis funera* on the host plant *Nothofagus truncata* was the only one among the seven major fungi that showed seasonal variation (Ruscoe, 1971). *Cladosporium cladosporioides* and *Epicoccum nigrum* on *Eucalyptus viminalis* leaves were the only two of the epifoliar fungi that showed seasonal variation with a minimum in summer that was positively related to humidity and inversely related to temperature (Cabral, 1985). However, since these studies did not consider the age of the leaves examined, it is unclear what this difference is really due to. In a study that analysed the factor seasonality within one year and the factor leaf age of epi- and endophytic fungi of *Camellia japonica* (Osono, 2008),

only some of the epiphytic fungi, viz. *Pestalotiopsis* sp., *A. pullulans*, *Phoma* sp., *Ramichloridium* sp. and *C. cladosporioides* varied with season. However, as *C. cladosporioides* and *A. pullulans* showed different patterns on different hosts due to leafage- and seasonal variation (Osono, 2008), it is difficult to decide which factor has the greatest influence. To study succession and distinguish it from seasonality, a study design is needed where different old plant substrates can be studied in parallel at a given time of year.

In the present project, this question was first investigated with an experimental design. On one site within the same experimental plot, SM was investigated on field-grown deciduous plants with ANL and perennial branches (Flessa et al., 2012). On a second site within the same plot, they were investigated on evergreen plants with CYL and PYL. In a second study, these aspects were investigated on an alpine evergreen plant in its natural habitat (Flessa and Rambold, 2013). Chomnunti et. al (2014) hypothesised that it is likely that there are specialised fungi for different stages of the SM mycobiome, such as initial pioneers and secondary specialists followed by the climax or steady state community. To date, however, there are almost no studies addressing this question. One aim of this project was therefore to compare SM mycobiomes on deciduous host plants with ANL and evergreen host plants with perennial leaves. Community composition of different age stages on evergreen leaves was also investigated. *A. pullulans* on *Salix* sp., *C. cladosporioides* on non-native evergreen hibernated host plants, and *C. rhododendri* on *R. ferrugineum* can be categorised as pioneers, while all other associated species on older plant structures can be assigned to the category of secondary fungi (Flessa et al., 2012; Flessa and Rambold, 2013). Pioneer species may be more host plant specific than associated fungi. This could also explain why SM fungi have higher host specificity than epiphytic mycobiomes on SM-uninfested leaves.

Two fungi were the predominant ones in Flessa et al. (2012) and showed an pattern of occurrence linked to the foliar factor. For instance, *A. pullulans* was present mainly on ANL and CYL, but was rare among the PYL samples, and *C. cladosporioides* was pre- or co-dominant on perennial tissues (Figure 7). Comparison of ANL, CYL, PYL and stems revealed significant differences in community composition between perennial leaves and ANL, as well as ANL and stems. There was no significant difference between CYL and PYL of evergreens, which is consistent with the results from studies on the transmission of plant pathogens from one reservoir to the next plant in the neighbourhood (Beckstead et al., 2010; Power and Mitchell, 2004; Wilson et al., 2014). SM mycobiomes as microbial communities on leaves may depend on the communities in their immediate environment, i.e. older leaves or stems, due to different dispersal mechanisms (e.g. airborne spores, rain, or vectors such as aphids and scale insects). For deciduous plants, the effect of short-distance dispersal is thought to have a greater influence, as the leaves of those plants are new and therefore recolonized each spring (Fort et al., 2016). However, this is not fully supported by the study of Flessa et al (2012), where fungal communities on stems, as the most obvious spore pool for new leaves, differ significantly from the established community on these ANL (Figure 7). This is mainly due to *Phoma* sp. and *C. cladosporioides* which are common in SM biofilms on twigs, whereas they were only found sporadically on ANL. *A. pullulans* seems

to be able to successfully colonise new substrates as a pioneer, but in older communities it is outcompeted by *C. cladosporioides*. However, the fact that *C. cladosporioides* also dominates on CYL draws attention on the aerial spore pool. Both host plant groups in this study were in the same geographic area, but with one major difference: the ANL and stems from deciduous trees were exposed to the 'natural' conditions, i.e. seasonal climatic variations, and airborne spore pool throughout the year. This is only partially the case with the perennial leaves: during the growing season they were exposed to the identical conditions as ANL, but during winter, the natural climatic conditions were excluded because they were brought into a greenhouse for overwintering.

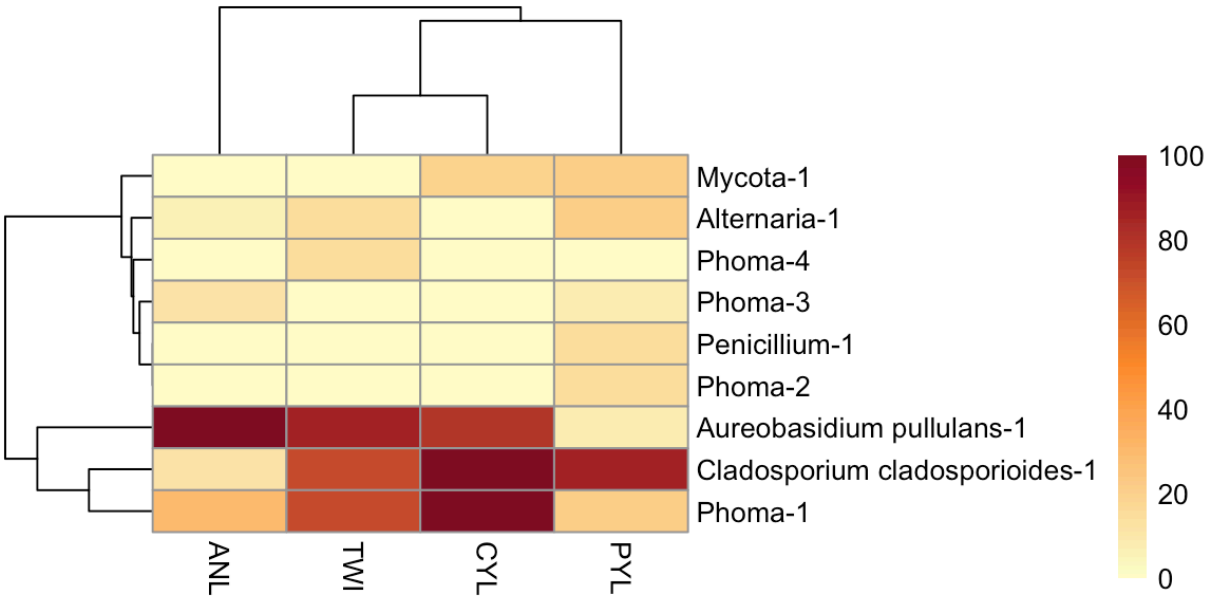

Figure 7 Heatmap of the most common taxa. Based on their steadiness in SM biofilms of annual (ANL), current year's (CYL) and previous year's (PYL) leaves of evergreens, and twig (TWI) samples. The colour scale illustrates the relative proportion of samples (%) in which the fungi were present. The data originate from Flessa et al. (2012), and the heatmap was created with the package 'pheatmap' in R (Kolde, 2019; R Core Team, 2020)

Therefore, the airborne spore pool seems to be decoupled from that of the ANL for the months of overwintering. In a suburb of Turin, the airborne spore pool was monitored for one year (Marchisio et al., 1997). When looking on the relative loads, *Cladosporium* has a high level throughout the year, but *Aureobasidium* spore loads are high only from February to July. If this is not just a local result for Turin, the exclusion of the airborne spore pool at the beginning of the year and the high spore load due to SM mycobiomes on leaves from the previous year could be a likely explanation for the differences between SM mycobiomes on ANL and CYL in the present project (Flessa et al., 2012). A higher concentration of aerial *A. pullulans* spores in the months prior to and during the period of increasing SM biofilm occurrence on willow leaves could explain the predominance of this fungus on ANL. This is an indication that in SM mycobiomes, which consisted mainly of ubiquitous fungi, the surrounding spore pool has a major influence on the community composition. Further studies are needed that continuously

measure the composition and concentration of the airborne spore pool in parallel with the composition of SM biofilms.

As the design in this particular study includes overwintering in a greenhouse, the relevance of the results obtained for SM mycobiomes under natural conditions is questionable. *R. ferrugineum* L., which is widespread from the European Alps to the Pyrenees (Crane et al., 2004; Ozenda, 1985) at altitudes from 1,600 to 2,200 m (Escaravage et al., 1998) is an ideal natural pendent to the study design in Flessa et al. (2012). As an evergreen shrub it offers the possibility to compare CYL with PYL. Moreover, due to snow cover during winter season, the leaves are only exposed to variations in climate and the airborne spore pool during the growing season. Snow cover during winter decouples the spore pool from the airborne pool above the cover and creates a more or less constant microclimate, which is comparable to the effect of a greenhouse on SM mycobiomes from CYLs leaves in Flessa et al. (2012). Due to snow cover, the leaves of *R*. f*errugineum* are exposed to mild temperatures close to 0 °C even in winter, and winter desiccation or freezing damage does not occur (Neuner et al., 1999). *R. ferrugineum* usually survives only at sites with reliable snow cover. Somewhat unexpectedly, however, a significant difference was found between CYL and PYL, which differs from the result of the CYL and PYL in the evergreens of the first study. *C. rhododendri*, the most dominant fungus on CYL, exhibits a dramatic decline in the PYL community (Figure 8). On the other hand, the second most dominant fungus, *Cladosporium* sp. shows a reverse pattern. Exclusion of the airborne spore pool cannot be the explanation for this result. It is more likely that a specific age-related variation in host leaf physiology is the determining factor for the observed increases or decreases in SM biofilm compositions. In *R. ferrugineum*, leaf glands activity changes dramatically during leaf ageing – from highly productive on young leaves to decreasing on older leaves (Flessa and Rambold, 2013). The factor of the airborne spore pool can be overridden by the change in the host plant-dependent nutritional composition, from which one of fungus of the community, viz. *C. rhododendri,* benefits most. The relationship of glandular secretions to nutrients available on leaf surfaces is obvious. Many host plants do not provide such additional nutrient sources. Thus, the absence of such glands may lead to an initial SM biofilm, driven by airborne spore pools, which is similar to what was observed in the first study.

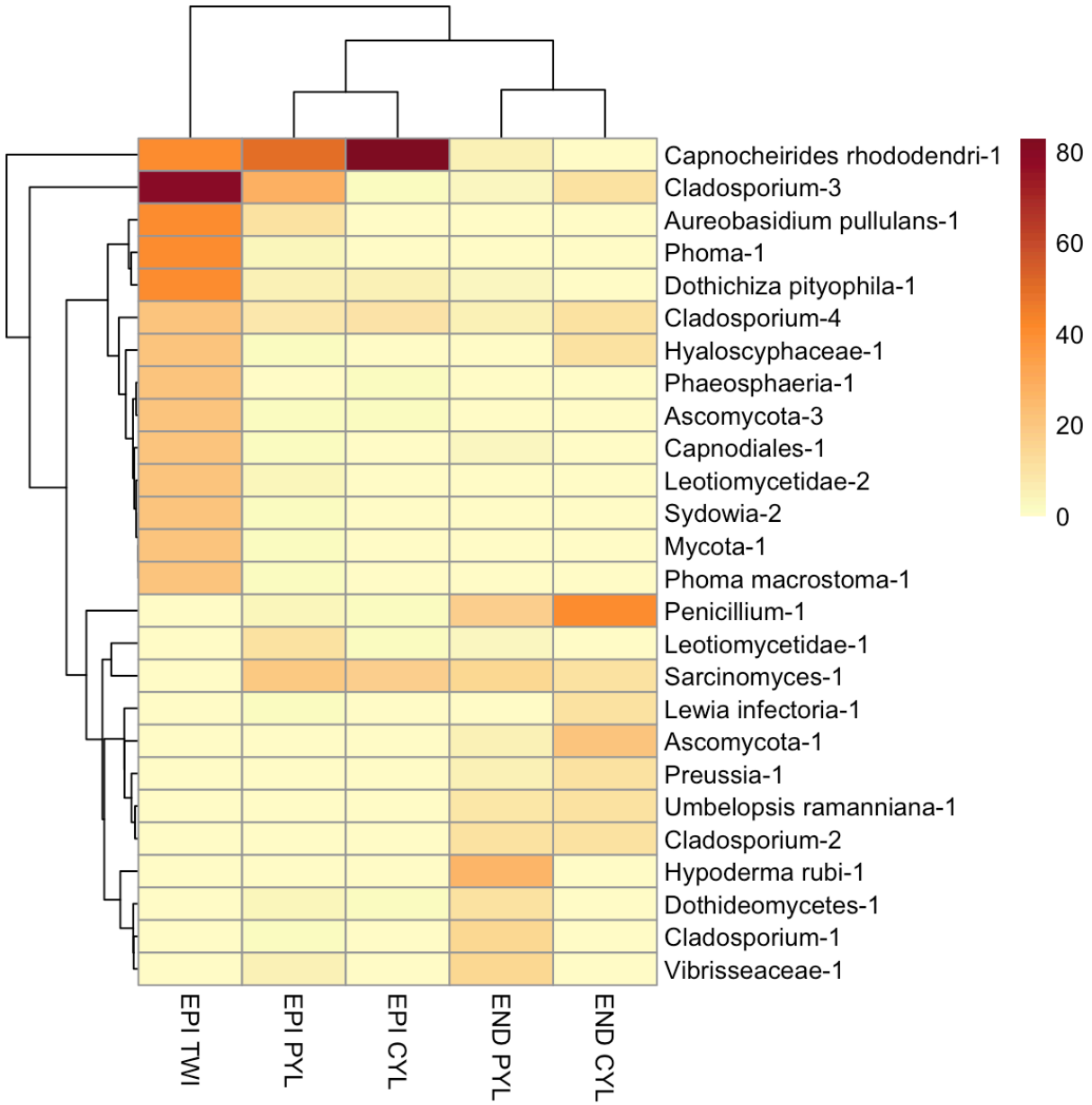

Figure 8 Heatmap of the most common taxa. Based on their steadiness in the epifoliar SM mycobiome (EPI) of current year's (CYL) and previous year's (PYL) leaves, twig (TWI) samples and endofoliar communities (END) on *R. ferrugineum* host plants. The colour scale illustrates the relative proportion of samples (%) in which the fungi were present. Data are from Flessa et al. (2013) and additional sequences of SM biofilms on twigs not used in the publication are listed in the Appendix (Table 1). The heatmap was created with the package 'pheatmap' in R (Kolde, 2019; R Core Team, 2020)

4.1.2 The steady state in sooty mould biofilms

For a steady state in the community composition of a fungal community, it is essential that the specific habitat is stable, i.e. provides unchanging conditions. This can be the case in abiotic environments such as buildings (Segers et al., 2016). However, as a biotic substrate, the leaf surface changes

during its lifespan. One of the most important age-related factors is surface roughness, which is higher in older leaves (Mechaber et al., 1996). The process of roughening takes place like geological erosion. The topography of the surface affects several key factors for epifoliar communities. It affects surface wettability, temperature, humidity, wind speed and it can also affect the localization of plant compounds on the leaf surfaces that are released from cells (Derridj et al., 1989; Ford and Salt, 1987). These changes may cause alterations of the SM mycobiome between younger and older plant structures. The surface of a leaf as a biotic and highly dynamic habitat does not provide a suitable environment for a steady state in fungal community composition. Furthermore, seasonal climatic variations and exposure to UV radiation increase the complexity of the habitat. A steady state cannot develop on ANL because the life span of the substrate is too short (Flessa et al., 2012). The maximum of SM biofilm development is reached on these leaves just before the leaf fall in autumn, but the SM biofilm would continue to develop if the leaves survived the winter. On twigs of *Salix* sp., *C. cladosporiodies* and *Phoma* sp. are found in 80% of the samples and may be considered as representatives of the steady state community. SM biofilms were rarely found on twigs of *R. ferrugineum*. In these, *C. cladosporiodes* is found in 100% of the samples, while all others are found in less than 50% of the samples (not included additional data from the study by Flessa and Rambold 2013, shown in the Appendix Table 1 and Figure 8). It is possible that the pioneers are rather specialists, the secondary fungi are a mixture of these pioneers and first representatives of the steady state community, while the steady state fungi are ubiquitous generalists, which is certainly true for *C. cladosporiodes*. There may also be fungi that are specialised to intermediate states (post-pioneer and pre-steady state), which could be the case for *Sarcinomyces* sp. on *R. ferrugineum*, which is somewhat less common on CYL than on PYL, but does not play a dominant role on twigs. However, with so many factors influencing the habitat and nutritional sources of SM mycobiomes on plants, which vary seasonally or over time, it is questionable how relevant the concept of a steady state really is for SM mycobiomes on plants and especially leaves.

4.2 Characterization of the core community

4.2.1 Co-occurrent, predominant and associated fungal species

As plants grow, fresh leaves provide new habitats that can be colonised by microorganisms of all kinds. Although ecological concepts fit well to macroorganisms, they are not necessarily transferable to microoganismic systems. One ecological concept that was applied early in the field of phyllosphere ecology by Andres and Kinkel (Andrews and Kinkel, 1986) is the theory of island biogeography (MacArthur and Wilson, 1967). Although unlike an emergent island, the leaf surface provides biological compounds as nutritional sources for microorganisms from the beginning of its existence. ‘Immigration’

is the arrival of a new species on a leaf (corresponding to a island) that is not yet colonised by the species. This species must be able to establish itself and reproduce on the leaf. Airborne spores or transmitted by another vector but cannot grow on the leaf surface are therefore excluded. The process by which species disappear through death or emigration is called 'extinction'. The term 'colonization' in the present context can be based on two different definitions. Either all species that land on an 'island' are included (Simberloff and Wilson, 1969) or only those that can establish and live on the island (MacArthur and Wilson, 1967). In the case of phylloplane fungi, two groups should be distinguished, as two pairs of terms exist: 'residents' and 'casuals' (Leben, 1965) and 'transients' and 'residents' (Andrews and Kinkel, 1986). According to Leben's definition, residents can multiply on the surface of healthy leaves without affecting the host. Casuals land on the surface, but are unable to grow (Leben, 1965). Transferred to the microcosm in an SM biofilm, it is very difficult to decide whether a detected fungus is only there by chance or whether it can actually grow on the surface. If an SB approach is chosen as opposed to a MB approach, it can be ensured that only living fungi are detected. However, only some of the fungi can be cultivated, and quantification is not possible with an SB approach. A fungus detected through SB could still simply originate from a fungal spore that does not contribute to the composition of the SM mycobiome. Therefore, other useful concepts are those of the core community for fungi present in at least 1/3 of all samples and associated fungi (Flessa et al., 2021b). In the core community, a further distinction can be made between dominant and co-occurrent fungi (Flessa et al., 2012; Flessa and Rambold, 2013). These categorizations do not imply the assumption that must be made when applying the definitions of Leben (1965) or Andrews and Kinkel (1986).

Co-occurrence or even promotion of one fungus by a second within one habitat is well documented by several studies on endophytic communities, e.g. *Xylaria* and *Guignardia* in leaves of *Coffea arabica* (Santamaria and Bayman, 2005). On the other hand, when they co-occur, antagonism is not far away: *Xylaria*, which is favourable for *Guignardia* can restrict the growth of *Colletotrichum*. The study by Santamaria & Bayman (2005) does not answer the question whether the reason for this pattern is antagonism or only the fact that some conditions on different leaves are more favourable for *Xylaria* and *Guignardia* than for *Colletotrichum*. Antagonistic effects between endophytic fungi have been shown for *Chaetomium* against *Curvularia lunata* in leaves of *E. citriodora* (Kharwar et al., 2010), *Chaetomium globosum*, *Penicillium chrysogenum* and *Streptomyces* in the host plant *Oryza sativa* (Naik et al., 2009). There is evidence of co-occurrence of *C. cladosporioides* with *A. pullulans* in the present project. *C. cladosporioides* is more common when *A. pullulans* occurs on the same leaf (Flessa et al., 2012). However, no co-occurrence was observed in samples from *R. ferrugineum* and the Brazilian rainforest site, but in samples from the Caatinga site in Brazil, Capnodiaceae sp. and *Capnodium* sp. tend to co-occur.

The dispersal of phylloplane fungal species occurs passively by rain flushing or other vectors (Andrews and Kinkel, 1986), such as leaf-sucking insects. A common fungal species found in various studies all over the world is *C. cladosporioides*, which is known to produce the greatest amount of wind-

born conidia in summer (Dickinson, 1981). Others, however, e.g. *Lasiothyrium* sp., produce spores only during the cooler months or are not affected at all by seasonal changes in their spore production, e.g. *Trimmatostroma* species (Goh and Yipp, 1996). If one observes effects of leaf senescence on the SM mycobiome living on it, the boundaries between predominant and associated fungi become blurred. *A. pullulans*, *C. cladosporioides* and *Phoma* sp.-1 occur together on CYL, but on PYL most SM biofilms are caused only by *C. cladosporioides*. *A. pullulans* decreases during the ageing process on leaves (Flessa et al., 2012). If just the SM biofilms from a host with evergreen leaves have been examined, *C. cladosporioides* would have been classed as a predominant species. The result is different on the leaves of deciduous host plants, which were sampled in the same area. Thus, there is a dominance of *A. pullulans*, and *C. cladosporioides* occurs only as an associated species.

On *R. ferrugineum* L., *C. rhododendri* is predominant (65% among all samples; 83% in CYL and 50% in PYL samples) and is accompanied on CYL and PYL by *Sarcinomyces* sp. (17% on CYL, 19% on PYL samples). But on PYL, *Cladosporium* sp.-3 outcompetes *Sarcinomyces* sp. and lies between these two fungi, being present in 29% of all samples (compared to 2% of CYL samples). For the period of monitoring, *C. rhododendri* can be classed as part of the core community and at the same time as the only predominant fungus on CYL and PYL, but the chances are high that if the SM mycobiome had been studied on a third generation of leaves, *C. rhododendri* would have been displaced by *Cladosporium* sp. This is also supported by the results in SM biofilm findings on twigs of *R. ferrugineum*, where *Cladosporium* sp. occurs in 80% of the samples, but *C. rhododendri* and all other fungi only in a maximum of 40% (Figure 8). The core community may thus change over time and consists of a different predominant species on the oldest substrates, while more fungi are found on older substrates. The concept of (pre-)dominant and associated fungal species thus always refers to a specific observation time and a group of samples from a defined habitat.

The species of the core community are habitat dependent. In the tropical core community from Caatinga examined with a MB approach, Capnodiaceae sp. and *Capnodium* sp. were predominant (94% of the samples). Other dominant fungi were *M. tassiana* (81%), a not further assignable Fungi sp. (63%), Capnodiales sp.-1 (56%), *Antennariella placitae*, Capnodiales sp.2, *Pestalotiopsis biciliate*, Phaeosphaeriaceae sp. *Toxicocladosporium strelitzia, Umbilicaria* sp. and *N. foedans* (all 38 – 44%).

In the core community from the tropical rainforest site, *N. foedans* was predominant, but only in 63% of the samples, together with *M. tassiana* (56%), *Umbilicaria* sp., *Erythrobasidium* sp. and *Strelitziana* sp. (all 38%).

Conclusions on objective III

On leaves of evergreen plants with ubiquitous dominant fungi, community composition does not differ significantly between this CYL and PYL at constant nutrition source. However, SM biofilms on leaves of evergreen plants differ in composition when the nutrition source changes considerably between young and old leaves. When host glandular activity ceases in older leaves, this significantly alters the SM mycobiome.

Predominant fungi in initial communities are A. pullulans *on ANL of native host plants in the temperate colline zone,* C. cladosporioides *on non-native evergreen plants overwintered in a greenhouse and* C. rhododendri *on* R. ferrugineum *in the European alpine region.*

SM mycobiomes on host plants with ubiquitous fungi separated from the spore pool of plants in the field in winter by overwintering in a greenhouse, differ significantly in the composition of SM mycobiomes on young leaves from leaves of the same age on deciduous plants in the field.

Due to the considerable changes of the leaf as a habitat during ageing, and the influence of seasonal climate fluctuations, there is no climax or steady state in SM biofilms on leaves.

The SM mycobiome consists of the core community, which includes fungi present in at least 1/3 of the sample group. These can be classified as predominant and co-occurring. All fungi that occur in less than 1/3 of the samples belong to the associated fungi.

The term predominant and associated fungal species always refers to a specific observed time and a clearly defined group of SM biofilms.

5 Objective IV: Rule of hyphal pigmentation

Among the cultivable fungi in the comparative study between SM mycobiomes from Europe and South America, 46% of all fungi were dark pigmented. Within the core community, this proportion rises to 60%, and among fungi occurring in half of the samples per site, 100% were dark pigmented (Flessa et al., 2021b). Melanin in black or dark pigmentation is essential for stress tolerance. Melanins from dematiaceous ascomycetes and deuteromycetes exhibit absorption maxima in wavelength ranges of 300 to 400 nm (ultraviolet), 600 to 700 nm (red), and 700 to 900 nm (NIR) (Babitskaya and Shcherba, 2002; Bell and Wheeler, 1986). They are composed of indoles and tyrosine intermediates that normally function to absorb light and provide protection from radiation damage in both fungi and animals (Riley, 1998). These pigments can help reduce mechanical or chemical stress caused by radiation or drought by sequestering metal ions or scavenging free radicals, and are associated with antimicrobial activities (Cordero and Casadevall, 2017) by protecting against cell lysis by cell wall-degrading enzymes of other microorganisms. Pigments embedded in the cell walls of dark pigmented ones make them more stable against mechanical stress than those of the light pigmented ones (Krah et al., 2019) These properties of

melanin are responsible for the predominance of melanised fungi on exposed sites such as plants or other surfaces (Kim, 2016).

In insects, there is a strong correlation that melanin is increased at high UV radiation (Clusella Trullas et al., 2007; Willmer and Unwin, 1981). However, this relationship does not exist in fungi. There were more light pigmented saprotrophic fungi especially in summer, when UV radiation was higher, and more dark pigmented fungi in winter, during the cold season (Krah et al., 2019). UV radiation was not found to be the significant contributor to pigmentation of ectomycorrhizal fungi and saprophytes, i.e. non-lichenised fungi, in the study by Krah et al (2018). The theory of thermal melanism (Clusella Trullas et al., 2007) states that dark pigmented ectothermic organisms have an advantage at low temperatures because they can warm up more easily. This theory is well tested in ectothermic animals, but the function of colours is largely unknown in the fungal kingdom (Caro, 2017; Cuthill et al., 2017; Krah et al., 2019; Sherratt et al., 2005). In yeasts, the dark pigmentation increases the absorption of radiant heat, which affects fitness at ambient temperatures and influences their latitudinal distribution (Cordero et al., 2018). Melanin is costly to synthesise (Eisenman and Casadevall, 2012) and therefore pigmentation is thought to increase fitness. Saprotrophic fungi have been the subject of a large-scale study in Europe and have been found to be darker in cold environments (Krah et al., 2019), supporting the theory of thermal melanism. Experimental tests with cultures of a dark and light pigmented variant of *Agaricus bisporus* showed that within 5 minutes, the dark pigmented culture warmed up by 1.2 °C more than the light pigmented one (Krah et al., 2019). This could be one of the reasons why *C. rhododendri* predominates on CYL leaves, as the snow cover allows sunlight to pass through even in winter. The very dark pigmentation of the slow-growing *C. rhododendri* could therefore give it an advantage over its competitors. By comparing the relative proportion of core community fungi in South America and Europe (Flessa et al., 2021b), the theory of thermal melanism was confirmed for the first time in SM biofilms. The lowest proportion of dark pigmented fungi was observed in the core community from the Brazilian rainforest (48%). The proportion was slightly higher in the Caatinga (59%), and a considerable increase was again observed between the European colline temperate region (67%) to the alpine region (100%).

A comparison of the pigmentation of SM mycobiome and endophytic fungi of *R. ferrugineum* (Flessa and Rambold, 2013) shows that common SM mycobiome fungi are almost exclusively dark pigmented, whereas this proportion is lower in endophytic fungi (Figure 9 total). This is consistent with mycobiomes on *Olea europaea* leaves: the epiphytic mycobiome of SM-uninfested *Olea europaea* leaves was dominated by fungal species with melanized hyphae and/or spores, whereas in the endophytic community the non-pigmented fungal groups were more abundant (Gomes et al., 2018). Both fungal communities on *R. ferrugineum* were found on the same plant with the same cold climatic conditions. However, it can also be assumed in this case that the protection from UV radiation is not the driving factor, as SM biofilms on this host plant are very often found on the underside of leaves and less frequently on the branches, but never on the upper surface of the leaves. As melanin production is very costly, increased pigmentation is expected in habitats where more carbohydrates are available, for

example in the ectomycorrhiza by the plant partner (Krah et al., 2019). However, this cannot explain the difference between the pigmentation of the endophytic and the SM mycobiome in *R. ferrugineum*, since sufficient carbohydrates are available in both habitats, as young leaves of *R. ferrugineum* have leaf glands that produce a high amount of glandular secretions.

In addition, seasonality in pigmentation was observed by Krah et al. (2018): saprotrophic fungi were darker in the colder periods of a year, and there is a significant effect of precipitation totals on fungal colour lightness of saprotrophs (Krah et al., 2019). A greater number of fruiting bodies were found in dark pigmented ones in winter, while the peak of light pigmented ones was in summer (Krah et al., 2019). Seasonality in the two communities can only be indirectly inferred for *R. ferrugineum* due to the study design chosen, as all leaves were collected in the summer months. While the fungi on and in leaves from the current year have not yet undergone winter, this cannot be the case for the fungi on leaves from the previous year. Pigmentation in the SM mycobiome is not affected by the age of the leaves, but for the endophytic fungi, a higher proportion of pigmented fungi was found in PYL than in CYL, regardless of the medium used (Figure 9).

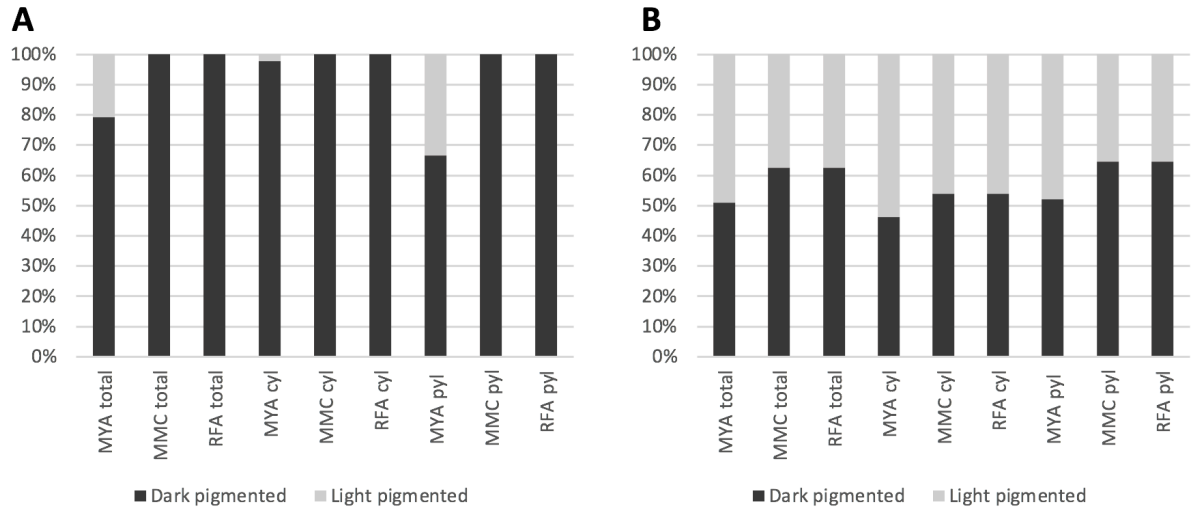

Figure 9 Pigmentation amounts of the most common fungi in the SM mycobiome (A) and endophytic community (B) on and in leaves of *R. ferrugineum*. Fungi were grouped according to pigmentation, based on the relative amounts of samples in which they were found. Total: overall samples; CYL: current year's leaves; PYL: previous year's leaves; MYA: malt-yeast-agar; MMC: Minimum agar medium with 4% carrot juice; RFA: agar medium with 20.75 g crushed fresh leaves of *R. ferrugineum* per litre. Only fungi that were present in at least 10% of the samples in either cyl or pyl per group were considered.

It is well documented that the degree of pigmentation of fungal cultures depends on the medium (Blechert et al., 2019; Kowalski et al., 2016; Palacio-Barrera et al., 2019). Examples of the variability of SM mycobiome fungi of this project in culture are shown in Figure 10. It must be considered that pH, carbon, and nitrogen source have a great influence on pigment production in fungal strains (Lathadevi et al., 2014), so pigmentation may differ in culture and *in situ*. Most of the common fungi showed dark pigmentation on one of the media used, with media containing crushed *R. ferrugineum* leaves or 4% carrot juice resulting in a higher level of pigmented cultures than only 4% carrot juice. In contrast, the proportion of fungi showing no pigmentation is lowest on a malt-yeast-glucose (MYA) full medium. However, every fungus that was darkly pigmented on MYA was also darkly pigmented on the other two media. When SM mycobiome fungi are examined for pigmentation, it can be assumed that the proportion of darkly pigmented fungi on an MYA medium is underestimated. Six of the fungi investigated, namely Ascomycota sp., *A. pullulans*, Leotiomycetidae sp., *Sirococcus conigenus*, *Coleophoma empetri*, *Cladosporium* sp. and Vibrisseaceae sp. were apparently able to adapt melanin production to the available nutrition and are facultatively pigmented, i.e. they do not show dark pigmentation on all three media. *Physalospora* sp., *Preussia* sp., and *Penicillium* sp. do not seem to be able to produce dark pigments at all. However, 13 fungi did not regulate their melanin production, most notably *C. rhododendri* and Sarcinomyces sp., which are the most common fungi in the SM mycobiome. It remains open whether they are unable to adjust their melanin production. Pigmentation is one of the most important factors for pioneers as well as for species that are the stronger competitors among the fungi of the SM mycobiome.

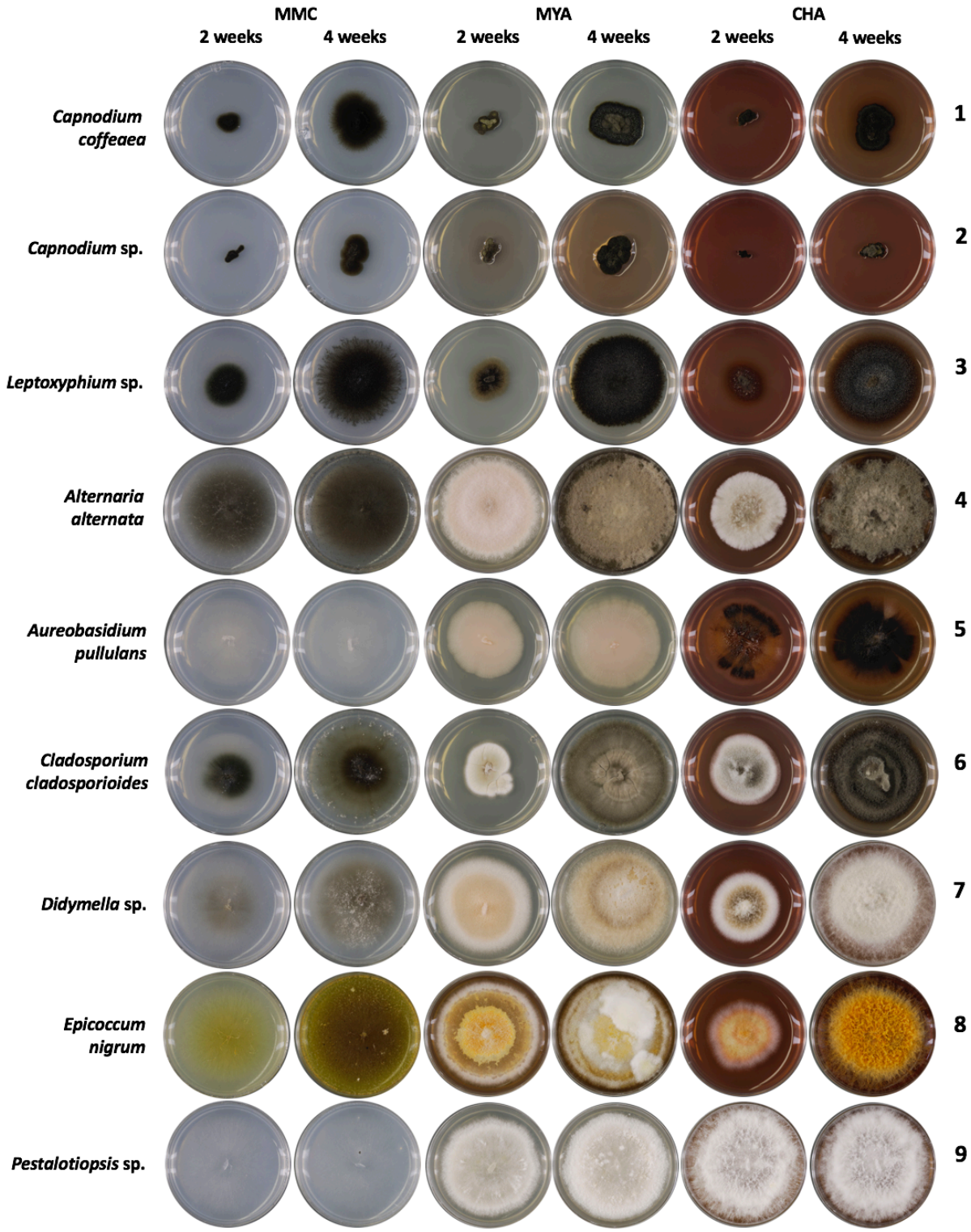

Figure 10 A exemplary selection of SM mycobiome fungi from this project. 1–3: Members of the Capnodiaceae, i.e. SMs in a taxonomy-based definition, 4–9: Fungi outside the SMs that belong to the SM mycobiome. All fungi grew at room temperature and were photographed under standardised conditions (Pietrowski et al., 2012) after two weeks and four weeks. MMC: Minimum agar medium with 4% carrot juice; MYA: malt-yeast-agar; CHA: agar medium with 20% cherry juice

Conclusions on objective IV

The proportion of darkly pigmented fungi is considerably higher at colder sites than at warmer ones

The pigmentation of the fungi within the core community confirms the theory of thermal melanism.

Members of the SM mycobiome are darkly pigmented in a higher proportion than those of the endophytic mycobiome on the same host plant. Among the fungi of the SM mycobiome, there are those that are facultatively pigmented and others that cannot regulate their pigmentation on different media.

6 Objective V: Link between epifoliar sooty mould biofilm and the endophytic fungal community

Less than a millimetre away from the epifoliar habitat, in the inner tissues of the host plants, is a completely different habitat: that of endophytic fungi. Besides spore pools, e.g. airborne spores or fungi sporulating near the host plant, which are two possible host plant-external sources of invaders into the SM mycobiome, endofoliar fungi could hypothetically also act as a reservoir for colonisation of the leaf surface and thus the SM mycobiome. The field of endophytic fungal communities has been investigated in several studies, but there are only a few of them comparing endophytic with epiphytic mycobiomes on SM-uninfested plant tissues on the above-ground plant parts based on morphological, SB or MB methods. Prior to the study of this project (Flessa and Rambold, 2013), no research has been conducted on a possible link between SM mycobiomes and endophytic communities.

The proportion of fungi identified using SB and found exclusively in the epiphytic, endophytic or both communities on leaves from six studies (Flessa and Rambold, 2013; Gomes et al., 2018; Kharwar et al., 2010; Mariano et al., 1997; Osono, 2007a, 2008) are summarised in Figure 11. Of these, only one treat SM mycobiomes, while all others are based on communities on and in leaves uninfested by SM. The proportion of fungi present in both communities, i.e. endophytic and epiphytic, is < 30% in all studies and < 20% in SM mycobiomes. The relative amount of exclusively epiphytic fungi is equal to or higher than that of endophytic fungi.

There is no difference between leaves of evergreen plants such as *Camellia japonica*, *Cocos nucifera*, *Eucalyptus citriodora*, *Olea europaea*, *Rhododendron ferrugineum* and the deciduous plant *Cornus stolonifera* with regard to this ratio. Also climatic zones, e.g. tropical (*C. nucifera*), subtropical (*C. japonica, E. citriodora, O. europaea)* and temperate *(R. ferrugineum, S. cinerea, C. stolonifera)* or geographical locations such as North America (*C. stolonifera*), South America (*C. nucifera*), Europe (*O. europaea*, *R. ferrugineum*, *S. cinerea*), and Asia (*C. japonica*, *E. citriodora*) have no influence on this ratio. While the proportion of fungi found in both habitats remains about the same between CYL

and PYL, the ratio of epiphytic fungi in the SM mycobiomes to endophytic ones shifts from younger to older leaves toward the epiphytic fungi on *R. ferrugineum*.

Epi- and endophytic fungal communities on leaves uninfested with SM and leaves with SM mycobiomes were significantly separated based on their species composition (Flessa and Rambold, 2013; Gomes et al., 2018; Kharwar et al., 2010; Osono, 2008, 2007b). Although both habitats are in close proximity to each other, this is not surprising. Epiphytic fungi in the phyllosphere are under a different selective pressure than endophytic fungi (Rastogi et al., 2013).

This overall comparison of fungal species says nothing about whether fungi that occur in both habitats are present with equal frequencies in the epi- and endophytic communities. In *R. ferrugineum,* the fungi belonging to this group that reached >10% abundance in either the epi- or endofoliar samples, namely *Capnocheirides rhododendri* and *Cladosporium* sp.-3 both were much more abundant in the epifoliar community, while *Cladosporium* sp. 1 and -2, *Hypoderma rubi*, *Penicillium* sp. and Vibrisseaceae sp. showed an opposite pattern, with only one fungi, *Sarcinomyces* sp. being equally abundant in both habitats (Flessa and Rambold, 2013). In a study of fungal communities in and on SM-uninfested *Coffea arabica* leaves, the four most common fungal genera were present in both communities, but also with an asymmetry: *Botryosphaeria* and *Pestalotia* were much more common as epiphytic ones, and *Xylaria* and *Colletotrichum* were mostly endophytic and only rarely epiphytic (Santamaria and Bayman, 2005). In a study on the phyllosphere of *Eucalyptus citriodora*, only one of the unspecific fungi, *Cladosporium cladosporioides*, was found in >10% of the samples, with a strong asymmetric pattern in favour of the epiphytic habitat (Kharwar et al., 2010).

The SM mycobiome on *R. ferrugineum* is significantly influenced by leaf age and sampling site, whereas the endofoliar fungal community is mainly influenced by altitudinal zone, and sampling site (Flessa and Rambold, 2013). *C. rhododendri* on *R. ferrugineum* leaves decrease drastically with leaf age, but is more abundant in the endophytic communities in older leaves, while it is completely absent in the endofoliar community in younger leaves (Figure 8). *Cladosporium* sp.-3 shows an opposite pattern, with a large increase beyond fungi in the SM mycobiome by a factor higher than ten from younger (2.4%) to older leaves (28.8%), but with the opposite pattern when the development in the endofoliar habitat is regarded. *Penicillium* sp. decreases in the endofoliar fungal community, but without a concurrent increase in the SM mycobiome. Such different patterns are also known from other studies. The effect of leaf ageing on migration from one habitat to the other can be seen in the most ubiquitous fungi that occur in both habitats in the phyllosphere on leaves of *C. stolonifera* uninfested with SM: *Colletotrichum gloeosporioides* is three times more abundant inside older leaves than in younger leaves, but twice as abundant on the phylloplane of younger than on older leaves (Osono, 2007a). In *C. japonica*, the predominant fungal endophyte, *C. gloeosporioides*, decreased with leafage, while *C. cladosporioides*, which was predominant among the epiphytic fungi, also increased with the age of the leaves (Osono, 2008). In the epifoliar fungal community of *R. ferrugineum*, the communities differed significantly due to factor leaf age, but not in the endofoliar community (Flessa and Rambold, 2013). In

contrast, no difference in the epiphytic fungal diversity was found between leaves and branches in *O. europaea*, although the difference was significant for endophytic fungi (Gomes et al., 2018).

The objection that there is a limitation of evidence due to the selective SB method used to obtain the data in these studies is justified. There are not yet many studies addressing the same question using MB methods. So far, there are only studies of fungal communities on leaves uninfested with SM, but none of SM mycobiomes. Yang et al. (2016) showed in their study of endofoliar and epifoliar fungal communities of the subalpine *Betula ermanii*, that the proportion of shared fungi is higher compared to culture-based approaches (Appendix Figure 1). Epi- and endophytic fungal communities were significantly different in their species composition (Yang et al., 2016). Another study investigated the community composition of epiphytic and endophytic fungi in six tropical mangrove plants (Yao et al., 2019). The community composition of the epiphytic and endophytic fungi differed in all plants, while the epiphytic fungi did not differ significantly in five plants, except for *Excoecaria agallocha*. In contrast, the compositions of the endophytic communities were significantly different among all plants. In each of the plants, the proportion of fungi found, both epi- and endophytic, was remarkably high. As both studies do not include the foliage factor, no conclusions can be drawn as to whether the asymmetric patterns found in association with leafage are also found using culture-independent methods. In both studies, it is noticeable that the proportion of shared fungi is significantly higher than in SB studies. In MB studies, the removal of epiphytic fungi is critical. If they are only inactivated, HTS still detects fungi as HTS does not distinguish between living or dead fungi. These are then incorrectly counted as shared fungi. Consequently, the proportion of fungi that occur in both habitats can be overestimated and the proportion of purely epiphytic fungi is greatly underestimated. According to an evaluation of the effectiveness of different surface sterilisation methods in MB-based fungal endophyte studies, physical treatments are more effective and reliable, while chemical treatments remove epiphytic fungi less efficiently (Burgdorf et al., 2014). In both MB-based studies cited, the leaves were only treated chemically, but not with physical methods before the endophytic fungi were analysed. It can therefore be assumed that the effect is method-based and that the shared fungi were overestimated in the cited MB-based studies.

Even though the overall ratio of shared fungi to strictly epi- and endophytic fungi is method-dependent, it is obvious that epi- and endofoliar fungal communities are interconnected to some extent. This applies to both SM mycobiomes and epiphytic communities on SM-uninfested leaf surfaces. The understanding of endophytic fungi as a reservoir for epiphytic fungi is controversial discussed. While colonisation of fresh leaf surfaces is immediate, colonisation of the endophytic habitat takes longer (Osono, 2008) and it is therefore unlikely that endophytic fungi migrate from the inner tissues of the leaf into the epiphytic community. Kharwar et al (2010) consider it possible that the epiphytic fungi influence the endophytic community and vice versa. Gomes et al (2018) suggest that phyllosphere endophytes may be former epiphytes that have invaded plant tissue. In our study on *R. ferrugineum*, we suggested that epiphytic fungi migrate into plant tissue. In this case, leaf glands could act as gateways (Flessa and Rambold, 2013).

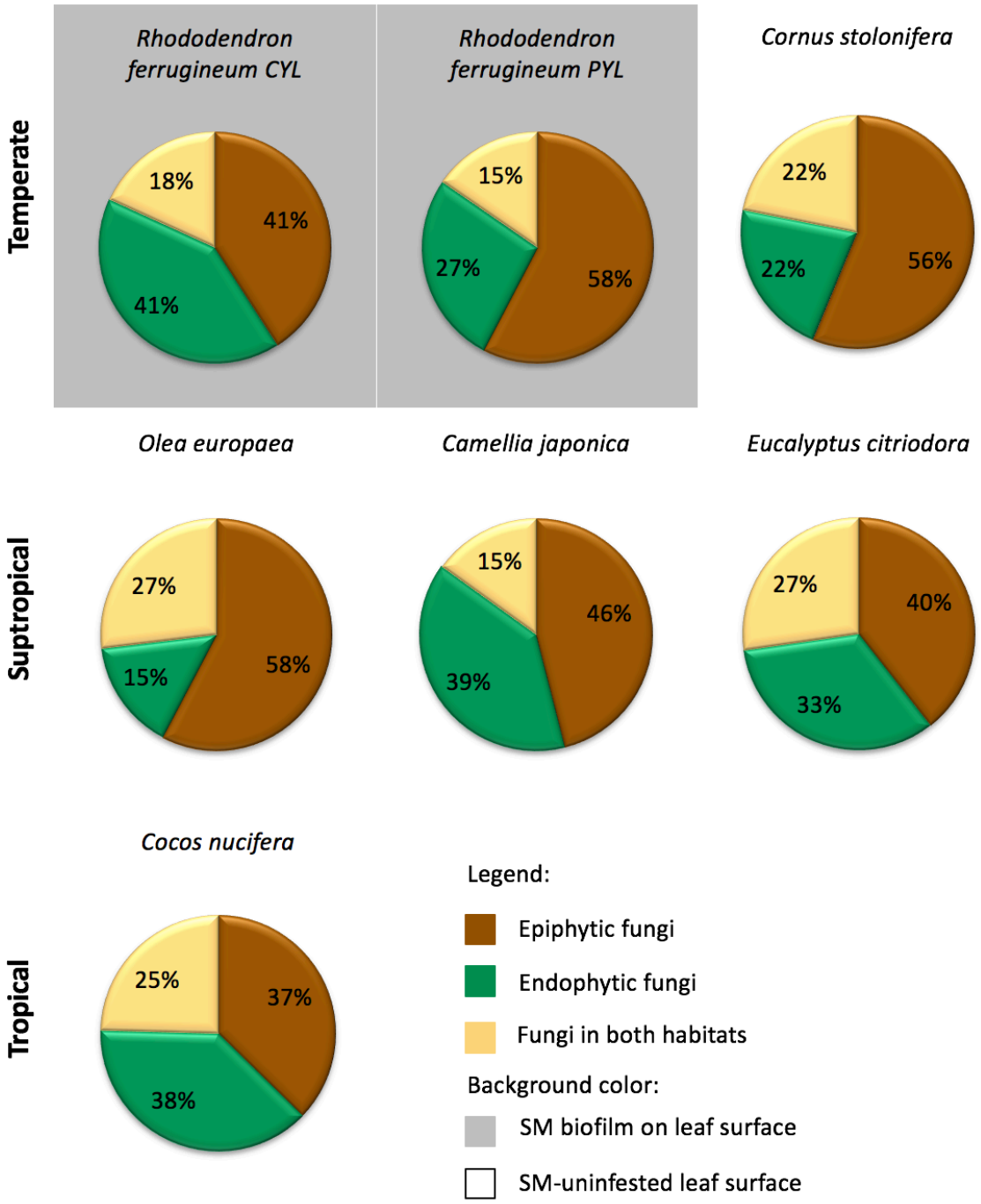

Figure 11 Relative amounts of exclusively epiphytic fungi, exclusively endophytic fungi, and fungi common in both communities in the phyllosphere of leaves from *R. ferrugineum*, *Cornus stolonifera*, *Eucalyptus citriodora*, *Camelia japonica*, and *Cocos nucifera*. All results were obtained through SB or morphological analyses.

Conclusions on objective V

SM mycobiomes are composed of at least as many, if not more exclusive fungal species than endophytic communities.

Less than 1/3 of the fungi occur in both habitats, in R. ferrugineum *the proportion is only 15-18%.*

While SM mycobiomes are more affected by leaf senescence and differ significantly between CYL and PYL, endophytic fungi on R. ferrugineum *are more influenced by altitudinal zone and site.*

It is more likely that fungal species migrate from the SM mycobiome to the inner leaf tissue during the process of leaf senescence than otherwise.

7 Objective VI: Prediction model

Although there are quite a number of studies on the influence of different factors on the composition of SM biofilms, only one study has so far investigated which factors promote or prevent them on a plant (Shukla et al., 2017). The effect of SFIs, grasshoppers and different weather conditions on the occurrence of SM biofilms has been studied over an extended period in the tropics on mango, an evergreen plant. As only one plant has been studied, there is still a lack of knowledge about the impact of different plant characteristics. Furthermore, there are no results yet on which factors influence the occurrence on deciduous host plants with ANL in a temperate region. In the present project, the occurrence of SM biofilms on 147 individuals of different species of the genus *Salix* (willows) during one growing season was investigated (Flessa et al., 2021a). The study area corresponded to the Salicetum, a sub-area of the study site of Flessa et al. (2012). However, for this study, all willows in the study area were monitored, regardless of whether they already had SM biofilm in the preceding study. Not all plants are necessarily host plants, even if the weather conditions are favourable. SM biofilms were found on slightly more than one third of the plants, but most individuals remained symptom-free during the observation season of the study.

Even if there is a time gap between SFI infestation and SM biofilm occurrence, significantly more SM biofilms can be observed on plants with SFIs on four or more observation dates. The infestation is 3.7 times higher.

Of the variables studied, namely: presence of SFIs, summer leaf position, smooth or rugose summer leaf structure, glabrous leaf blade, light- or dense-haired, glaucous, flat, concave or convex adaxial leaf vein type, adaxial midrib type, adaxial midrib pubescence, visible adaxial surface gloss, leaf length and form, leaf glands, surface waxes, those that were significant ($p < 0.05$) or almost significant ($p < 0.1$) in univariate models were selected for a multivariate model. Subsequently, the variables with the lowest p-values were successively removed from the model, resulting in a model with SFI observations on four or more dates, horizontal leaf position, and concave leaf veins. The relative abundance of SM biofilms

predicted by the model was 35% (CI 15–63%). In comparison, the relative abundance predicted by the model on trees with SFIs <4, no horizontal leaf position and sunken leaf veins was 1.2% (CI 0.5–3.1%). The multivariate plant model was then extended with individual weather variables. The weather variables that, in combination with the plant model, resulted in the highest (>80%) predicted relative abundances of SM biofilms were a mean vapour pressure deficit (VPD) of 3 hPa at 8:00 a.m. over three consecutive preceding days, increased wind speed of 2.1 m/s over the last three days, a high relative humidity of 90% at 8:00 a.m. over the last 14 days, a high precipitation sum of 85–90 mm over the last 14 days, and high relative humidity at 8:00 p.m. of 91% over the last 7 days. Of the weather parameters, the ones that had the greatest effect in this study were those that led to higher moisture on the leaf surfaces. The availability of water is an important factor for fungal growth (Ruinen, 1961). If the wettability of a leaf changes during senescence, it affects the fungal growth by facilitating germination and the growth of germ tubes (Cabral, 1985; Dickinson, 1986, 1981). The occurrence of SM biofilms cannot be attributed to favourable weather conditions alone, as favourable weather conditions only led to an increased probability of occurrence when they occurred in combination with prolonged or repeated SFI presence and favourable plant characteristics.

An important result of this study is that a temporary, short-term SFI presence has no significant effect on the occurrence of SM biofilms. Only with SFI observations on four or more observation dates could a significant effect be observed. Thus, SFI infestation in crops should be controlled consequently, as prolonged presence of SFIs leads to increased prevalence weeks after SFIs have disappeared. In greenhouses, good moisture management is also important to prevent SM biofilm growth. Breeding plants with less SM-promoting characteristics could also help reduce infestation.

Conclusions on objective VI

SFIs also significantly influence SM biofilm infestation on deciduous trees with ANL. Among the host plant characteristics, infestation with SFIs (≥ 4 observation dates), horizontal leaf position and leaves with sunken veins have the strongest effect on the occurrence of SM biofilm.

Among the weather parameters, those leading to higher moisture on leaf surfaces had the strongest influence in this study, i.e mean VPD of 3 hPa at 8:00 a.m. in the last three consecutive preceding days, increased wind speed of 2.1 m/s over the last three days, high relative humidity of 90% at 8:00 a.m. over the last 14 days, high rainfall total of 85–90 mm over the last 14 days, and high relative humidity at 8:00 p.m. of 91% in the last 7 days.

8 Methodology applied

8.1 Advantages and limitations of cultivation-based and cultivation-independent approaches

Studies on fungal communities are currently mainly conducted by high-throughput sequencing (HTS) (Bao et al., 2019; Beng and Corlett, 2019; Checinska Sielaff et al., 2019; Chen et al., 2019; Egidi et al., 2019; Epstein et al., 2019; Ezeokoli et al., 2020; Li et al., 2019; Lynikiene et al., 2020; Morales-Rodriguez et al., 2019; Nerva et al., 2019; Qian et al., 2020; Ricks and Koide, 2019; Saravesi et al., 2019; Wilkinson et al., 2019; Würth et al., 2019; Yao et al., 2019), but there are very few that focus on SMs (Dhami et al., 2013; Flessa et al., 2021b). Although many studies currently rely solely on MB data, there are also recent studies that are based on cultivation to identify fungi in a community (Berlanas et al., 2019; Bien and Damm, 2020; Chen et al., 2020; Gomes et al., 2018; Janowsky et al., 2019; Martins et al., 2021; Oh et al., 2020; Salamon et al., 2020; Santos et al., 2019). A few studies use both approaches in parallel (Dissanayake et al., 2018; Jayawardena et al., 2018; Mbareche et al., 2020; Mendoza et al., 2017; Zapka et al., 2017) or in a supplementary manner (Flessa et al., 2021b; Janakiev et al., 2019; Kraková et al., 2018).

SB approaches have some limitations. In general, many fungi cannot be cultivated because they are biotrophic, and not all cultivable fungi grow on all types of culture media, so these factors already have a selective effect (Yang et al., 2001). This effect can be exploited if only the saprotrophic part of the fungal community is to be studied, since the isolated fungi species are non-biotrophic or partially biotrophic (Flessa et al., 2021b). However, some known biotrophic fungi can also grow saprotrophically, e.g. *Botryosphaeria dothidea* and *B. cinerea*, so a SB approach does not exclude all biotrophic fungi (Dissanayake et al., 2018). Due to their biotrophic habits, efforts to cultivate strictly biotrophic species have not been successful (Hansford, 1961; Hosagoudar, 1996; Vitoria et al., 2010) and were therefore not included in the SB analysis of the present project. However, their presence is taken into account in the MB approach.

Especially for studies in SM mycobiomes, which are defined as non-parasitic, it is necessary to distinguish the saprotrophic or potential saprotrophic from the strictly biotrophic fungi. This is useful in the study on SM mycobiomes from South America (Flessa et al., 2021b), as the parasitic black mildews (Meliolaceae) that grow on leaves in the tropics (Hansford, 1961; Hosagoudar, 1996) have similar traits to the pigmented saprotrophic species, such as the degree of pigmentation and hyphal growth, can co-exist within the SM mycobiomes analysed. Host plant species for Meliolaceae in Brazil are known to belong to the Anacardiaceae, Asteraceae, Euphorbiaceae, and Fabaceae (Macedo et al., 2010; Pinho et al., 2012, 2009; Silva et al., 2012). These host plant families overlap with the host range of SM mycobiomes in this project.

Some fungi are able to sporulate in culture, which means that counting the number of colonies from environmental samples does not lead to reliable results regarding abundance (Clay et al., 2016; Steinrucken et al., 2016). Therefore, only the steadiness among individual samples was evaluated in SB approaches (Flessa et al., 2021b, 2012; Flessa and Rambold, 2013), but not the number of colony-forming units. It is also possible that very slow-growing or low-competitive species are overlooked in the SB (Chomnunti et al., 2014), although they could actually be cultivated. Therefore, in a SB approach, it is important not to use only one standard method, such as 14 days of growth on MYA medium at room temperature, but to test different approaches if possible. For the SM fungus *C. rhododendri*, preliminary tests for the study of Flessa and Rambold (2013) showed that it can only be reliably isolated from environmental samples if the culture was allowed to grow at 15 °C for one month (Flessa and Rambold, 2013), as its growth rate decreases at higher temperatures. In this case, cultivation at room temperature for 14 days would have resulted in it not being detected at all or only rarely, and another fungus, probably *Cladosporium* sp., would have been falsely identified as predominant, as it grows much faster at higher temperatures.

A major advantage of the SB approach is that the fungi are actually isolated in culture and not only a very short gene fragment is available as in MB approach. This allows studies to be carried out on isolates of individual species of a community, such as on pigmentation (Flessa et al., 2021b; Flessa and Rambold, 2013), on growth rate, enzymatic activity and on the production of secondary metabolites. This in turn leads to a deeper understanding of interactions within a community that cannot be achieved by determining diversity alone. Moreover, if these isolates are deposited in public culture collections, new strains are available for the study of useful and much-needed compounds, such as new antibiotics, as was discovered some time ago in a culture of an SM fungus, *Capnodium* sp. (Herath et al., 2012), or to create more complete fungal phylogenies that include SM members. Currently, only a few deposited cultures of sooty fungi are available. Of the SM families within the Dothideomycetes, e.g. Antennulariellaceae, Capnodiaceae, Euantennariaceae and Metacapnodiaceae, only Capnodiaceae cultures were considered in a recent large-scale current study (Abdollahzadeh et al., 2020).

MB approaches have the advantage of covering non-cultivable fungi, but simultaneously have disadvantages when it comes to identification of the fungi. Many of the sequences cannot be identified down to genus or species level (Maharachchikumbura et al., 2011; Wikee et al., 2011), and many of the sequences deposited in the GenBank are associated with the wrong taxonomic names. Many species groups cannot be distinguished based on ITS or other parts of rDNA, which is especially relevant for Ascomycota. Furthermore, many fungi have not yet been sequenced (Crouch et al., 2009; Dissanayake et al., 2018; Nilsson et al., 2015, 2012). Taxonomic results obtained from HTS are likely only reliable only down to genus level (Dissanayake et al., 2018). Therefore, studies often assigned them only to genera, families, orders or classes (Abdelfattah et al., 2015; Dhami et al., 2013; Izuno et al., 2016; Qian et al., 2020; Würth et al., 2019; Yang et al., 2016; Yao et al., 2019). Sequences obtained by HTS are often associated with taxa not reported in previous studies and with sequences not linked to any fungal

sequences in GenBank (Ko Ko et al., 2011; Taylor et al., 2016; Tejesvi et al., 2010), so the proportion of fungi that cannot be further identified is quite high (Dissanayake et al., 2018). The dominant *C. rhododendri* in SM mycobiomes of this project could already be assigned to this fungus at the time of the study by SB procedures based on microscopic determination of the culture material, which was not possible at that time by using database queries based solely on ITS fragment sequence data in GenBank, as no reference sequence of this species was available. Even today, there are entries for this fungus in UNITE at genus level only (Nilsson et al., 2019).

HTS of DNA does not distinguish between inactive or dormant (representing the past or future) and active members (representing the present) of the community (Laforest-Lapointe and Whitaker, 2019). This is problematic for SM mycobiomes, as the proportion of spores deposited simply randomly could be overestimated, even if they are no longer viable and therefore do not contribute to the structure of the SM biofilm. In studies investigating endophytic and epiphytic fungal communities on different host plants (Yang et al., 2016; Yao et al., 2019), the proportion of fungi present in both habitats was estimated to be many times higher than in comparable SB studies (Flessa and Rambold, 2013; Gomes et al., 2018; Kharwar et al., 2010; Osono, 2008, 2007b). In such studies, effective removal of epifoliar fungi is necessary. It is not sufficient that they are no longer viable, as discussed in chapter 6.

In HTS studies, it is common to assign guild-affiliation to fungal taxa via FUNGuild (Nguyen et al., 2016). Since sequence assignment is only done by assignment via taxon names, a potentially incorrect assignment can also lead to an incorrect affiliation. For 11 of the 27 very common cultivable SM mycobiome ascomycetes, no saprotrophic nutrition type was registered in FUNGuild (Flessa et al., 2021b). Thus, if FUNGuild had been used solely for classification into a guild, more than 1/3 of these taxa would have been incorrectly classified as exclusively biotrophic. Especially when identifying a saprotrophic nutrition mode, it is therefore necessary that in addition to identification via HTS, a SB approach is chosen.

When a SB and MB approach was used in parallel, similar results were obtained with both approaches in terms of the significant factors (Dissanayake et al., 2018). This could also be confirmed for SM mycobiomes in this PhD project with regard to the significant results (Flessa et al., 2021b). In a direct comparison between MB and SB, a large proportion of the common fungi could be detected in both approaches (Dissanayake et al., 2018; Mbareche et al., 2020). Only individual species could be found using only one of the two approaches (Dissanayake et al., 2018; Jayawardena et al., 2018; Kraková et al., 2018; Mbareche et al., 2020; Mendoza et al., 2017).

For future research on SM mycobiome fungal communities, HTS technologies should be combined with general SB methods, allowing synchronized exploration of a more complete picture of fungal communities on host plants (Hardoim et al., 2015). HTS-based studies without reference cultures may be misleading and much of the data generated in the past needs to be critically considered (Dissanayake et al., 2018). This should be compensated with additional SB.

8.2 The use of the ITS for fungal identification

The internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal RNA gene cluster are the most commonly used nuclear markers for estimation of conspecifity in plants (Edger et al., 2014) and for fungal diversity studies (Dentinger et al., 2011; Mbareche et al., 2020; Roe et al., 2010; Schoch et al., 2012) based on the assumption that these markers are non-functional and therefore evolutionary neutral (Edger et al., 2014). Their widespread use is based on a number of criteria: ubiquitous presence in target taxa, sufficient sequence variation among taxa, ease of obtaining sequence data, the presence of a basic understanding of the function of the locus and the possible selective forces acting on its sequence evolution (Balajee et al., 2009; Blaalid et al., 2013; Edger et al., 2014; Mbareche et al., 2020). Since the early 1990s, it has been used extensively in both molecular systematics and ecological fungal studies (Blaalid et al., 2013). The ribosomal RNA gene cluster consists of seven components: the 5′ external transcribed spacer, the 18S rDNA exon, the internal transcribed spacer 1 (ITS1), the 5.8S rDNA exon, the internal transcribed spacer 2 (ITS2), the 28S rDNA exon, and the 3′ external transcribed spacer (Wheeler and Honeycutt, 1988). While the 5.8S gene is conserved, the ITS1 and ITS2 regions usually provide resolution within a genus and often to species level (Nilsson et al., 2008; Schoch et al., 2012). The ITS sequence length is highly variable between fungal species and depends on the primers used. It ranges between 450-900 bp (Blaalid et al., 2013; Mbareche et al., 2020; Toju et al., 2012b). However, in addition to the advantages mentioned, ITS has also limitations. Many species groups cannot be resolved using ITS (Balajee et al., 2009; Dissanayake et al., 2018; Gazis et al., 2011; Maharachchikumbura et al., 2011; Wikee et al., 2011) and there are known problems with the reliability of the ITS sequences deposited in reference databases (Bidartondo, 2008; Kõljalg et al., 2013; Nilsson et al., 2006). Nevertheless, there is considerable consensus regarding the use of ITS sequencing as the initial step in fungus identification (Balajee et al., 2009), as evidenced by its frequent use in recent studies of diversity in fungal communities (Bao et al., 2019; Beng and Corlett, 2019; Berlanas et al., 2019; Beule et al., 2019; Bien and Damm, 2020; Checinska Sielaff et al., 2019; Chen et al., 2000; Del Frari et al., 2019; Epstein et al., 2019; Flessa et al., 2021b; Gomes et al., 2018; Janakiev et al., 2019; Janowsky et al., 2019; Jayawardena et al., 2018; Li et al., 2019; Lynikiene et al., 2020; Martins et al., 2021; Morales-Rodriguez et al., 2019; Nerva et al., 2019; Oh et al., 2020; Qian et al., 2020; Ricks and Koide, 2019; Santos et al., 2019; Saravesi et al., 2019; Schiro et al., 2019; Wilkinson et al., 2019; Würth et al., 2019; Yao et al., 2019; Zhang et al., 2019). Despite the limitations of resolution that also apply to the SM mycobiome fungi, ITS is nevertheless suitable for addressing the main factors shaping the SM mycobiome (Chomnunti et al., 2014). A major problem is that the databases do not yet contain many of the known SMs (Chomnunti et al., 2014). Thus, the queries often do not result in a genus-level assignment. A few years ago, the deficit was so large that more than 50% of the SM mycobiome fungi were not identified even at the class level in a New Zealand study (Dhami et al., 2013). In the present project, 60% could be assigned at order level or higher, using an MB approach, one third even at species level, and 26% could not even be assigned to a phylum level (Flessa et al., 2021b).

In the HTS approach, either the ITS1 or ITS2 region is used, as the entire ITS region is too long to be fully sequenced by second generation sequencing (Li et al., 2020). Studies have revealed mixed assessments of the performance of ITS1 and ITS2 in documenting and characterising fungal biodiversity (Arfi et al., 2012; Bazzicalupo et al., 2013; Blaalid et al., 2013; Heinrichs et al., 2012; Ihrmark et al., 2012; Kelly et al., 2011; Kohout et al., 2014; Monard et al., 2013; Osmundson et al., 2013; Tedersoo et al., 2015b, 2015a). In recent studies, the use of the two ITS regions is quite inconsistent. While some use ITS2 (Bao et al., 2019; Beng and Corlett, 2019; Egidi et al., 2019; Epstein et al., 2019; Li et al., 2019; Lynikiene et al., 2020; Morales-Rodriguez et al., 2019; Nerva et al., 2019; Qian et al., 2020; Ricks and Koide, 2019; Saravesi et al., 2019; Wilkinson et al., 2019; Würth et al., 2019; Yao et al., 2019; Zhang et al., 2019), others based their studies on ITS1 (Beule et al., 2019; Checinska Sielaff et al., 2019; Del Frari et al., 2019; Flessa et al., 2021b; Schiro et al., 2019).

The ITS1 barcode yields higher richness and diversity scores than ITS2, implying that ITS1 is better able to recover more OTUs and better estimate the richness (Mbareche et al., 2020), but there were considerable differences between ITS1 and ITS2 in terms of species abundance between individual classes. Many of these differences may be due to the choice of different primers (Bellemain et al., 2010; Han et al., 2013; Tedersoo et al., 2015a, 2015b) or the presence of introns between primer sites of some fungal groups of the Ascomycota (Bhattacharya et al., 2000; Perotto et al., 2000). In a recent review, Nilsson et al. (2019) even go so far as to say that the primers dictate which fungi are found in the samples (Nilsson et al., 2019). In terms of the number of singletons, both barcodes are comparable and in terms of differences from different samples, similar results were obtained with both regions (Mbareche et al., 2020). ITS1 has a significantly lower GC content than ITS2 (Wang et al., 2015), which is an advantage for PCR and sequencing efficiency (McDowell et al., 1998). Unlike ITS2, ITS1 has the ability to capture taxonomic profiles similar to those obtained with shotgun metagenomic (Mbareche et al., 2020). For this reason, Mbareche et al. strongly recommend the use of ITS1 as the universal fungal barcode (Mbareche et al., 2020), as it has already been implemented in many studies (Beule et al., 2019; Checinska Sielaff et al., 2019; Chen et al., 2000; Del Frari et al., 2019; Flessa et al., 2021b; Schiro et al., 2019). For the two main problems, namely species split into several clusters or species clusters in one cluster, there is no difference between ITS1 and ITS2 (Blaalid et al., 2013), and this seems to apply to many lineages of the fungal kingdom (Gazis et al., 2011; Nilsson et al., 2008).

Studies using SB approaches are generally based on both ITS regions (Berlanas et al., 2019; Bien and Damm, 2020; Flessa et al., 2021b, 2012; Flessa and Rambold, 2013; Gomes et al., 2018; Janakiev et al., 2019; Janowsky et al., 2019; Martins et al., 2021; Oh et al., 2020; Santos et al., 2019) and thus provide better confidence in fungal sequences assignment.

The use of operational taxonomic units (OTUs) is also inconsistent versus the use of amplicon sequence variants (ASVs) in more recent studies. Although there are recommendations to use only ASVs (Callahan et al., 2017), leading to a reduction in the number of dominant species (Egidi et al., 2019), many of the current studies are based on OTUs (Albright et al., 2020; Bao et al., 2019; Beng and Corlett,

2019; Beule et al., 2019; Checinska Sielaff et al., 2019; Chen et al., 2019; Epstein et al., 2019; Garnica et al., 2020; Janakiev et al., 2019; Jia et al., 2020; Lynikiene et al., 2020; Morales-Rodriguez et al., 2019, p.; Nerva et al., 2019; Pan et al., 2019; Park et al., 2020; Qian et al., 2020; Ricks and Koide, 2019; Saravesi et al., 2019; Wilkinson et al., 2019; Würth et al., 2019; Yang et al., 2020; Yao et al., 2019; Zhang et al., 2019), others are based on ASVs (Del Frari et al., 2019; Deyett and Rolshausen, 2020; Egidi et al., 2019; Li et al., 2019; Lynum et al., 2020), or OTUs generated by subsequent grouping from ASVs (Ezeokoli et al., 2020; Flessa et al., 2021b). The advantages of ASVs are that they capture all variations of the marker gene in a dataset and ASVs derived from a specific dataset can be reproduced and validly compared in future datasets (Callahan et al., 2017). But ASVs also have disadvantageous limitations. 100% sequence similarity can lead to false discrimination between single nucleotide polymorphisms (SNP) of the same species. This zero tolerance for sequence differences can lead to a very high number of ASVs per sample and consequently lead to missing information about the core microbiome (Mbareche et al., 2020). In the present project both, ASVs and on ASV-based OTUs, were used to prepare for publication of the study using a MB approach. What Mbareche et al. found was also observed in this case. Therefore, in this project, the advantage of ASVs over traditional OTUs was used by first generating ASVs and then building OTUs based on them.

9 Challenges for future research

There are still very few studies on diversity in SM mycobiomes using MB methodology. So far, there are only the studies by Dhami et al. (2013) from New Zealand and Flessa et al. (2021) from the temperate colline region in Germany, the alpine region from Switzerland and a tropical region from Brazil. Further studies could help to better understand the mechanisms of succession, host plant dependence or nutrition source. However, MB methods should always be used in combination with SB methods when studying SM mycobiomes, as many SMs are not yet recorded in the databases, which may lead to an inaccurate assignment of the dominant fungal species. If an SB method has been used, the cultures should be deposited in public collections. This also provides the basis for future studies to better understand SMs. Another gap is that despite efforts to produce up-to-date phylogenies, not all SMs have been included in such studies. A good example is *C. rhododendri*, which is also missing form the most recent study (Abdollahzadeh et al., 2020) and for which assignment to a family has not yet been made (Roskov et al., 2019).

The impact of the airborne spore pool on the species composition of the SM mycobiome is indirectly implied in this project, but has not yet been investigated. A study that examines the composition of SM biofilms and available airborne spores in parallel would fill this gap.

The study comparing endophytic and SM mycobiomes on the same host plant was conducted using SB methods, but to date there are no results based on a MB approach.

The SM mycobiome is influenced and delimited by many different factors that have been weighted very differently in the history of research on epifoliar fungi. Some aspects were overlooked in previous studies because the study designs were not suitable to address a specific question, e.g. the impact of the host plant species or the strong dependence on honeydew as a nutrition source. The study in this project on factors that favour SM biofilms on willows in addition to SFIs was a first step towards identifying other factors besides SFIs that make a plant an ideal host plant for SM biofilms. Further studies are needed to identify these factors also in tropical regions where SM biofilms regularly cause economic damage by infesting crops.

That succession within SM biofilms is strongly influenced by the available nutrient source was shown in the SM mycobiome on *R. ferrugineum*, where young leaves produce additional glandular secretions that are not available in older leaves. The extent to which SMs in the strict sense dominate over ubiquitous ones when extra nutrient sources are available needs to be verified in SM mycobiomes on other host plants.

10 References

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11 Publications of this PhD project and declaration of own contribution to each manuscript

This PhD project contains six research articles to which I contributed as declared below.

Core manuscripts of this PhD project:

Manuscript 1:

Flessa F., Peršoh D, Rambold G. (2012): Annuality of Central European deciduous tree leaves delimits community development of epifoliar pigmented fungi. – Fungal Ecology **5** (5): 554-561.

Status: published
DOI: https://doi.org/10.1016/j.funeco.2011.12.005

Own contribution:

Concept of the study 30%, concept of the manuscript 50%. Fieldwork and culture work were done by me (100 %). Data analysis was done by me (100%) under guidance from Prof. Dr. G. Rambold and Dr. D. Peršoh. I wrote the manuscript (90%) taking the comments of all co-authors into account.

Manuscript 2:

Flessa F. and Rambold G. (2013): Diversity of the *Capnocheirides rhododendri*-dominated fungal community in the phyllosphere of *Rhododendron ferrugineum* L. – Nova Hedwigia **97**: 19-53.

Status: published
DOI: 10.1127/0029-5035/2013/0110

Own contribution:

Concept of the study 50%, concept of the manuscript 50%. Field collection of fungal material, isolation of fungal strains in single cultures and data analysis was done by me (100%). I wrote the manuscript (100%) taking the comments of my supervisor Prof. Dr. G. Rambold into account.

Manuscript 3:

Flessa F., Harjes J., Cáceres M. S., Rambold G.: Comparative analyses of sooty mould communities from Brazil and Central Europe. Mycological Progress **20,** 869–887.

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Own contribution:

Concept of the study 50%, concept of the manuscript 50%. Field collection of fungal material was done by Prof. G. Rambold and Dr. M. S. Cáceres in Brazil, and by me in Bayreuth and Switzerland. Culture work was performed by me (100%). DNA isolation of cultures was performed and sequencing of fungal OTUs and of host plant DNA with the assistance of the Technical Assistant (80%), HTS was performed by J. Harjes (Bayreuth). I wrote the manuscript (95%) under consideration of the comments of all co-authors.

Manuscript 4:

Flessa F., Kehl A., W. Babel, G. Rambold, M. Kohl (2021): Effects of sap-feeding insects, plant characteristics and weather parameters on sooty moulds in the temperate zone. Manuscript in preparation for submission to Oecologia.

Status: in preparation for submission

Own contribution:

Concept of the study 50%, concept of the manuscript 80%. Field data collection done in equal shares by A. Kehl, an undergraduate assistant and myself. Generalized linear mixed models were performed by Prof. Dr. Matthias Kohl (Hochschule Furtwangen University). I wrote the manuscript (100%) with consideration of the comments of all co-authors

Methodical manuscripts in the context of the Ph.D. project:

Manuscript 5:

Pietrowski A., **Flessa F.**, Rambold G. (2012): Towards an efficient phenotypic classification of fungal cultures from environmental samples using digital imagery. – Mycological Progress 11 (2): 383-393.

Status: published
DOI: https://doi.org/10.1007/s11557-011-0753-2

Own contribution:

Concept of the study 20%, concept of the manuscript 30%. Field collection of fungal material, isolation of fungal strains in single cultures and sequencing of all strains was performed by A. Pietrowski (University of Bayreuth) with the assistance of the technical assistant at the University of Bayreuth, Department of Mycology. I contributed to the conceptual design of the study by Prof. Dr. G. Rambold and carried out the lab work, i.e. culture work, the photography, and the analysis of the data obtained from culture pictures. Contribution to the text of the manuscript: 30%.

Manuscript 6:

Flessa F., Kehl A., Kohl M. (2013): Analysing diversity and community structures using PCR-RFLP: a new software application. – Molecular Ecology Resources **13**: 726-733.

Status: published
DOI: https://doi.org/10.1111/1755-0998.12094

Own contribution:

Concept of the study 80%, concept of the manuscript 80%. PCR-RFLP and Sequencing of strains for all datasets used were performed by me. Conceptual design of the package, programming of the RFLP-Package and the release in the CRAN-Project was performed by Prof. Dr. Matthias Kohl (Hochschule Furtwangen University). Tests and comments on the programming were performed by A. Kehl and me in equal shares. I wrote the manuscript, taking the comments of all co-authors into account.

12 Manuscripts of this PhD project

The manuscripts are sorted according to the order in the listing in Chapter 12. Appendices published as supplementary data are included directly after each manuscript.

12.1 Manuscript 1

Flessa F., Peršoh D, Rambold G. (2012): Annuality of Central European deciduous tree leaves delimits community development of epifoliar pigmented fungi. – Fungal Ecology **5** (5): 554-561.
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Annuality of Central European deciduous tree leaves delimits community development of epifoliar pigmented fungi

*Fabienne FLESSA**, *Derek PERŠOH*, *Gerhard RAMBOLD*

University of Bayreuth, Department of Mycology, Universitätsstraße 30, D-95447 Bayreuth, Germany

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ABSTRACT

Communities of dark pigmented epifoliar fungi of various deciduous tree species from habitats in the surroundings of Bayreuth (Upper Franconia, Germany) were compared to those of evergreen plants overwintered in a greenhouse. The fungi were mainly assignable to the genera *Aureobasidium*, *Cladosporium*, *Alternaria* and *Phoma*, with most plants hosting more than one fungal genotype or taxon. While a host preference was not detected among the fungal taxa, the community composition significantly differed between annual and perennial leaves. The results indicate that the epifoliar fungal community found on leaves of deciduous Central European trees represents an early developmental stage of the same, but further developed community, found on older evergreen leaves. We therefore assume that the life-span of deciduous leaves is too short for the fungal community to reach a steady state.

Introduction

Plant surfaces are often colonized by darkly pigmented epiphytic fungi (Schoulties 1980), which are commonly referred to as 'sooty moulds'. However, the usage of the term 'sooty mould' is rather inconsistent. Sooty moulds in the narrow sense are defined as pigmented epiphytic fungi that form dense mats on living leaves in the tropics (Kirk *et al.* 2008) or, in a wider sense, pigmented epiphytic fungi that live on the exudate of aphids and scales (Hughes 1976; Parbery & Brown 1986; Perez *et al.* 2009). Epiphytic microbiota is known to thrive on secretions of the host plant (Weyman-Kaczmarkowa & Pedziwilk 2001), or on deposited pollen grains (Fokkema 1984). The definition of 'sooty mould' can also be restricted to certain taxonomic groups (Hughes 1976).

Previous studies on pigmented epiphytic fungi mostly concerned fungal α-diversity and focused on single, mostly economically relevant, plant species (Webb & Mundt 1978; Tolstrup 1984; Tolstrup & Smedegaard-Petersen 1984; Perez *et al.* 2009). These investigations provided evidence that fungal communities forming sooty patches on leaves are mostly composed of oligo- or multi-species associations, in contrast with the single- to oligo-species communities formed by phytopathogens like rust and smut fungi and mildews (Parbery & Brown 1986; Perez *et al.* 2009). *Aureobasidium pullulans* is usually the most frequent species in communities of pigmented epiphytic fungi, but it is more or less regularly accompanied by representatives of a varying spectrum of genera such as *Fusarium*, *Alternaria*, *Epicoccum*, *Chaetomium*, *Cladosporium*, *Lewia*, *Phoma*, *Mucor* and *Rhizopus* (Webb &

* *Corresponding author*. Tel.: +49 921 55 2455; fax: +49 921 55 2567.
E-mail address: fabienne.flessa@uni-bayreuth.de (F. Flessa).
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Mundt 1978; Fenn *et al.* 1989; Yang *et al.* 2001; Osono 2002). While the reasons for the resulting differences in community composition are not known, host preferences of the fungi or a temporal sequence of successional stages seem to be plausible explanations.

The present study focused on the successional stages of pigmented epiphytic fungal communities on a relatively wide range of angiosperm host plant species by comparing the communities on deciduous and evergreen host species. The following question was addressed: how much does the spectrum of fungal taxa and genotypes correspond with the taxonomic relationships of the host plants, as is known for several phytopathogenic fungal groups (O'Kane 1910; Hasan 1974; Hughes 1976; Goos 1978; Francis 2002)? The central aim of the present study, however, was to clarify whether the ephemeral nature of the living leaves or the phylogenetic status of the host plants determines the community composition of fungal taxa. The particular goal of the study was to test the following hypotheses: (1) Sooty patches generally indicate the presence of a multi-partite fungal community or association. (2) The composition of pigmented epiphytic fungal communities in Central Europe is independent of the taxonomic affiliation of the host plant. (3) Major differences in fungal community composition exist between deciduous and evergreen plant species due to durability of the host leaves.

Material and methods

Conceptual design of the study

To address the question of whether or not substratum durability of annual leaves is sufficiently high to allow darkly pigmented epifoliar fungal communities to reach a steady state, plants with perennial leaves were included in the study design. Because native plants with perennial leaves (e.g., conifers) in the study area (surroundings of the city of Bayreuth, Upper Franconia, Germany) lacked sooty patches, taxa with persistent leaves were included in the setup of the study. These were kept in a greenhouse during winter.

The epifoliar fungal community composition was analyzed on two groups of trees with sooty patches: (a) plants with annual leaves from the study area (Bayreuth University campus area, 49°55′43.11″ N, 11°35′4.44″ E, 352 m alt., inner city of Bayreuth, 49°56′58.41″ N, 11°34′52.75″ E, 347 m alt. and Salicetum of the Bayreuth University Ecological-Botanical Garden, 49°55′32.26″ N, 11°34′58.34″ E, 352 m alt.) and (b) mostly non-European angiosperm trees with various phylogenetic relationships and perennial leaves, which were cultivated and overwintered in a greenhouse at the Ecological-Botanical Garden of the University of Bayreuth, and placed outdoors during the growing season (49°55′29.70″ N, 11°35′3.73″ E, 352 m alt.). Since leaves of both deciduous and perennial species were exposed to similar climatic conditions during the growing season, the climatic factors that can affect epifoliar fungal communities (Talley *et al.* 2002) were excluded as reasons for potential growth and composition differences. Climate data were available from a weather station in the Ecological-Botanical Garden of the University of Bayreuth, near the Salicetum and the outdoor refugium of the trees that were overwintered in the greenhouse. The mean temperature and total precipitation for the sampling periods were: May-06 12.7 °C, 118.74 mm; Jun-06 16.6 °C, 31.96 mm; Jul-06 21.4 °C, 106.84 mm, Aug-06 14.7 °C, 67.34 mm; May-07 14.2 °C, 126.04 mm; Jun-07 17.6 °C, 96.95 mm; Jul-07 17.2 °C, 121.7 mm; Aug-07 16.6 °C, 54.18 mm.

Whereas annual leaves were only sampled from the actual vegetation period, perennial leaves were analyzed from the previous vegetation period in addition to the current year's perennial leaves. Fungal material was also sampled from the stems of deciduous plants, which represent a perennial substratum not protected during winter. This analysis allowed us to test whether or not cold hardiness is a driving factor for the development of different communities.

Host plants

All plants were exposed to the same conditions during the growing season. Leaves and twigs with sooty patches were collected from May to Jul. 2006 (stem, annual- and previous year's perennial leaves) and in autumn 2007 (current year's perennial leaves). One to three leaves or twigs were cut from each host plant, once.

Details of the host plants, including herbarium accession numbers and sampling dates, are given below. The nomenclature and classification of the host plants followed the Catalogue of Life Annual Checklist 2010 (http://www.catalogueoflife.org/annual-checklist/2010/search/all). Representative material was dried and deposited in the vascular plant collection at the Botanische Staatssammlung München (M).

Annual leaves
Eight species of *Salix*, grown in the Salicetum at the Ecological-Botanical Garden of the University of Bayreuth, without manipulation (application of pesticides or fertilizers, or truncation), were sampled: *S. appendiculata* (M-0128450), *S. atrocinerea* (M-0128448, 06.07.2006), *S. aurita* (M-0128455, 06.07.2006), *S. foetida* (M-0128454, 06.07.2006), *S. foetida* × *waldsteiniana* (M-0128451, 06.07.2006), *S. rugulosa* (M-0128452, 06.07.2006), *S. wallichiana* (M-0128449, 06.07.2006) and *Salix* sp. (M-0128453, 06.07.2006). Infected leaf material was also collected from eight plants grown under semi-natural conditions on the university campus, including three individuals of *Quercus robur* (M-0128439, 29.05.2006; M-0128436, 29.05.2006; M-0128444, 06.07.2006), and one individual of each of *Cornus mas* (M-0128460, 10.06.2006), *Corylus avellana* (M-0128458, 29.06.2006), *Q. cerris* (M-0128443, 06.07.2006), *S. cinerea* (M-0128457, 29.06.2006) and *Tilia cordata* (M-0128459, 29.06.2006)

Stems and twigs
Twigs were sampled from five species of *Salix* from the Salicetum. Two individuals of *S. nigricans* (M-0128435, M-0128438, both 29.05.2006) and one representative of each of *S. apennina* (M-0128441, 29.05.2006), *S. elaeagnos* (M-0128437, 29.05.2006) and *S. purpurea* (M-0128434, 29.05.2006) were included. Stems and twigs of *Rosa* sp. (M-0128422, 24.04.2006) and of *S. cinerea* (M-0128457, 29.06.2006) were collected in the inner city area of Bayreuth and from the surroundings of the Ecological-Botanical Garden, respectively.

Perennial leaves

Perennial leaves with sooty patches were analyzed from individuals of *Acca sellowiana* (M-0128429, 29.05.2006), *Aucuba japonica* (M-0128447, 06.07.2006), *Citrus limon* (M-0128465, 22.06.2006), *Coleonema album* (M-0128430, 29.05.2006), *Dietes grandiflora* (M-0128471, 29.05.2006), *Elaeodendron capense* (M-0128428, 29.05.2006), *E. orientale* (M-0128445, 06.07.2006), *Ilex latifolia* (M-0128456, 06.07.2006), *Lantana camera* (M-0128470, 29.05.2006), *Trachycarpus fortunei* (M-0128432, 29.05.2006) and *Yucca elephantipes* (M-0128431), and from two individuals of *Citrus* sp. (M-0128468, 29.05.2006; M-0128467, 29.05.2006). The plants were not treated with pesticides from the middle of Jun. until Sep.. During winter, the plants were placed in a greenhouse at 10 °C. No fertilizer was added during winter and, when necessary, the plants were treated with a plant protection agent against pseudococcids. From Jan. to Aug., mineral fertilizer was given three times and liquid fertilizer was added once per week. In addition, leaves were collected from one plant of *Buxus sempervirens* (M-0128440, 20.04.2006) from the inner city area.

Current year's perennial leaves

The five host plant species sampled: *A. japonica* (M-0128447, 01.06.2007), *C. album* (M-0128430, 01.06.2007), *E. capense* (M-0128428, 01.06.2007), *E. orientale* (M-0128445, 01.06.2007) and *I. latifolia* (M-0128456, 01.06.2007), were grown under the same conditions as described for the group of (older) perennial leaves.

Isolation of fungi and morphology-based determination

Fungi were isolated on the same day as the plant material was sampled. Pieces of mycelium were cut off from the infected twigs or upper leaf surfaces and washed in sterile tap water to remove most of the superficially adhering, airborne fungal spores. The pieces were transferred to Petri dishes (5.5 cm diameter) containing yeast-malt medium (4 g glucose, 10 g malt extract, 4 g yeast extract, and 12 g agar per litre) with 0.1 % tetracycline to suppress bacterial growth. The Petri dishes were incubated at room temperature and screened daily for mycelia. Outgrowing hyphae were transferred onto new plates until pure cultures were obtained. The consistency of the molecular name assignments with the morpho-anatomical concepts was assured for the most relevant taxa (*A. pullulans*, *C. cladosporioides*, *Alternaria* sp. and *Phoma* sp.) by light microscopic examinations of at least two cultures representing each cluster (see below). One fungal strain of each group with more than two members was deposited in the collection of the Fungal Reference Center Jena (PRZ Jena) under the accession numbers FSU8643 (corresponding sequence accession number: GU942896), FSU10093 (GU942923), FSU9943 (GU942921), FSU10094 (GU942922) and FSU10095 (GU942920). An assortment of singletons was also deposited: FSU8644 (GU942846), FSU6577 (GU942874), FSU6478 (GU942879), and FSU6479 (GU942886).

DNA extraction, amplification, sequencing and grouping of the ITS nrDNA

Pure cultures were sorted according to their phenotypic traits (i.e. the presence and type of aerial mycelia, growth form and type of pigmentation). From all strains isolated from each plant, at least one representative of each morphotype was chosen for sequencing. The Charge Switch® gDNA Plant Kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and the DNeasy Plant Kit (Qiagen, Hilden, Germany) were used for DNA isolation from culture material. Cell disruption was accomplished using the Fast Prep FP120 (Bio101, Thermo Fisher Scientific Inc., Waltham, MA, USA) at a speed of 6.0 m s^{-1} for 2×40 s. Double-stranded sequences of the ITS rRNA gene were obtained and further processed according to the method of Triebel *et al.* (2005). Sequences were deposited in the NCBI GenBank under the accession numbers GU942834—GU942923 (see Supplementary Table 1).

For statistical analysis, fungal isolates were grouped according to their ITS rRNA sequence similarities as described by Peršoh *et al.* (2010). Briefly, pairwise similarities among length-adjusted sequences were calculated using the BLAST application 'blastall' (v. 2.2.18). The resulting tabular output dataset was transformed with the function 'simMatrix', package 'RFLPtools' (Flessa *et al.* 2010) in R (R Development Core Team 2010). A cluster analysis was conducted using the function 'hclust' with the clustering method 'average linkage' in R, and clusters with minimal similarities of 96 % were grouped.

Nineteen strains were included according to their phenotypic traits only. The characteristics of these 19 cultures unambiguously corresponded to those of other isolates assigned to the respective groups according to the cluster analysis.

Sequence-based assignment of names to similarity groups

Taxonomic names were assigned to the clusters to allow a comparison with the results of other studies, but all data were exclusively analyzed on the basis of sequence clusters, independently of the assigned names. The nomenclature followed Index Fungorum (http://www.indexfungorum.org) and MycoNet (http://www.fieldmuseum.org/myconet). Names were assigned to the sequences, and correspondingly to the isolates, based on the nearest relatives provided by the 'Mega BLAST' (Zhang *et al.* 2000) results in the NCBI database (http://www.ncbi.nlm.nih.gov; status: Jan. 2010). A consensus name was compiled from the names under which sequences obtaining a 'bitscore' of at least 90 % of the best matching sequences were deposited, following the method of Peršoh *et al.* (2010). Details of the name assignment are listed in the Supplementary Table 1. Groups, i.e. clusters, were named according to the sequences included, with the consecutive numbering of groups having otherwise identical names.

Analysis of similarity (ANOSIM) served to further assess the reliability of the assigned names by analyzing the sequence similarity matrix (see above) against the classification linked to the assigned names.

Data analysis

The binary matrix coding the presence/absence of the fungi of each cluster in each sample was transformed into a similarity matrix based on Jaccard distances using PRIMER 6 (Plymouth Routines, v. 6.1.6). Similarities between fungal communities were visualized by non-metric multidimensional scaling

(NMDS) and by hierarchical cluster analysis with the group average method applied. The respective isolation source (annual or perennial leaf, stem and current year's perennial leaf), the sampling location, and the phylogenetic affiliation of the host plant taxon were coded as factors grouping the samples. The ANOSIM analysis was conducted to assess the impact of each factor on the grouping of the samples; ANOSIM calculates the *p*-value, which was considered to indicate significant differences when below 0.05. The *R*-value indicates to what degree the respective factors explained groupings among the samples; *R*-values > 0.75 were interpreted as indicating clearly separated groups, $R > 0.5$ overlapping, but clearly different groups, and $R < 0.25$ barely separated groups (Chapman & Underwood 1999).

To analyze possible effects of host plant phylogenetic affiliations on the composition of the pigmented epiphytic fungal communities, plant orders with only one representative plant individual (singletons) were excluded. Orders of plants with perennial leaves represented by at least two individuals were Sapindales with *Citrus* (three individuals) and *Coleonema* (1), and Celastrales with *Elaeodendron* (2). Annual plant orders represented by more than one individual were Salicales with *Salix* (10) and Fagales with *Quercus* (4), *Corylus* (1) and *Cornus* (1).

Results

Taxonomic affiliation of the isolates

Of a total of 99 strains, 80 of those that were sequenced were isolated from 37 plant individuals, representing 33 species. Of the 80 sequences, 69 were grouped into nine clusters by hierarchical cluster analysis and 11 represented singletons. Taxonomic names could be assigned at the species level to 27 sequences, the genus level to 45, family to three sequences, and order to one sequence. The remaining four sequences were classified as fungi that were not identifiable any further than 'Mycota'. The assigned names as well as information for estimating the reliability of the sequence-based name assignment are provided as Supplementary Table 1.

The ANOSIM analysis revealed that the factor 'taxonomy', i.e. the name assignment according to the BLAST search results, significantly explained ($p < 0.05$) the genetic dissimilarities among the sequences at the various taxonomic levels, i.e. at species level ($p = 0.002$, $R = 1.000$, $n = 27$), genus level ($p = 0.001$, $R = 0.996$, $n = 72$), family level ($p = 0.001$, $R = 0.994$, $n = 55$), and order level ($p = 0.001$, $R = 0.990$, $n = 76$). The *R*-value was only considerably lower ($p = 0.001$, $R = 0.654$, $n = 76$) at the subclass level. The most frequently occurring fungi in the samples analyzed were *A. pullulans*-1 (27 strains), *C. cladosporioides*-1 (23) *Phoma*-1 (17), *Alternaria*-1 (5), Mycota-1 (4), *Penicillium*-1 (2), *Phoma*-2 (2), *Phoma*-3 (2) and *Phoma*-4 (2). Singletons were excluded from further analyses.

Fungal preference of host plant substrata assessed by fungal abundance

Oligo-species communities were found for 28 samples, and single-species symptoms for 14 samples (Fig 1). Among the 15 samples from *Salix* investigated herein, four sooty patches were exclusively formed by *A. pullulans*-1 or *Phoma*-1 and 11 by at least two species. *Aureobasidium pullulans*-1 was present in all clusters that contained annual leaf samples, but infrequently occurred in the perennial leaf samples (7 %), which were regularly inhabited by *C. cladosporioides*-1. *Cladosporium cladosporioides*-1 mainly occurred on perennial leaves (86 %), on the current year's perennial leaves (100 %) and on stems of deciduous plants (60 %). *Aureobasidium pullulans*-1 and *C. cladosporioides*-1 co-occurred in 11 samples (26 % of all samples, 39 % of all samples with *A. pullulans*-1). *Phoma*-1 showed no distinct pattern of occurrence and representatives of the other groups were detected in less than 12 % of the samples.

The epiphytic fungal communities were just not significantly separated among the host plant species ($p = 0.054$, $R = 0.324$), but they were significantly separated among the host taxa at a higher level ('host plant genus': $p = 0.001$, $R = 0.447$; 'host plant family': $p = 0.001$, $R = 0.414$; 'host plant order': $p = 0.001$, $R = 0.441$). Significant pairwise differences in the epiphytic fungal community composition between host taxa are listed in Table 1. Global ANOSIM also revealed host plant phenology to be a significant grouping factor ($p = 0.01$, $R = 0.483$). The corresponding pairwise tests (Table 2) revealed significant differences between fungal communities on annual and perennial leaves ($p = 0.001$, $R = 0.773$), and annual leaves and the current year's perennial leaves ($p = 0.001$, $R = 0.491$). The stems only partly differed from annual leaves ($p = 0.005$, $R = 0.308$), and even less from perennial leaves ($p = 0.022$, $R = 0.287$) with respect to the fungal communities. Differences in the composition of pigmented epiphytic fungal communities on plant individuals with annual leaves ($n = 17$ plant individuals) and perennial leaves ($n = 14$ plant individuals) also became evident from the NMDS plot ordination (Fig 2). Both groups were already separated by differences in the presence of the most frequent fungal groups (Fig 3), which were *A. pullulans*-1 with 18 strains (annual:perennial leaves = 17:1), *C. cladosporioides*-1 (2:12), *Phoma*-1 (5:3) and *Alternaria*-1 (1:3).

No significant groupings were formed according to the factors 'sampling date' ($p = 0.33$, $R = 0.031$) and 'sampling month' ($p = 0.128$, $R = 0.051$). While 'host plant location' appeared to have a significant effect ($p = 0.001$) at first sight, the calculated *R*-value of 0.331 was lower than the *R*-value calculated for 'host plant phenology' ($R = 0.483$).

Discussion

Whereas only a fraction of microbial communities is usually covered by culture-based methods (Yang *et al.* 2001), this method was evidently sufficient for detecting the majority of symptomizing fungal strains because previous studies reported the same taxa in pigmented epiphytic fungal communities (Webb & Mundt 1978; Fenn *et al.* 1989; Yang *et al.* 2001; Osono 2002). Furthermore, substratum durability-dependent shifts in fungal community composition were detected, which indicates that the fungal spectrum isolated was sufficient for addressing the hypotheses focused on in this study. However, the factor 'host plant phenology' and 'host plant location' may not be statistically differentiated, because all evergreen trees were kept within (winter) or close to (summer) the

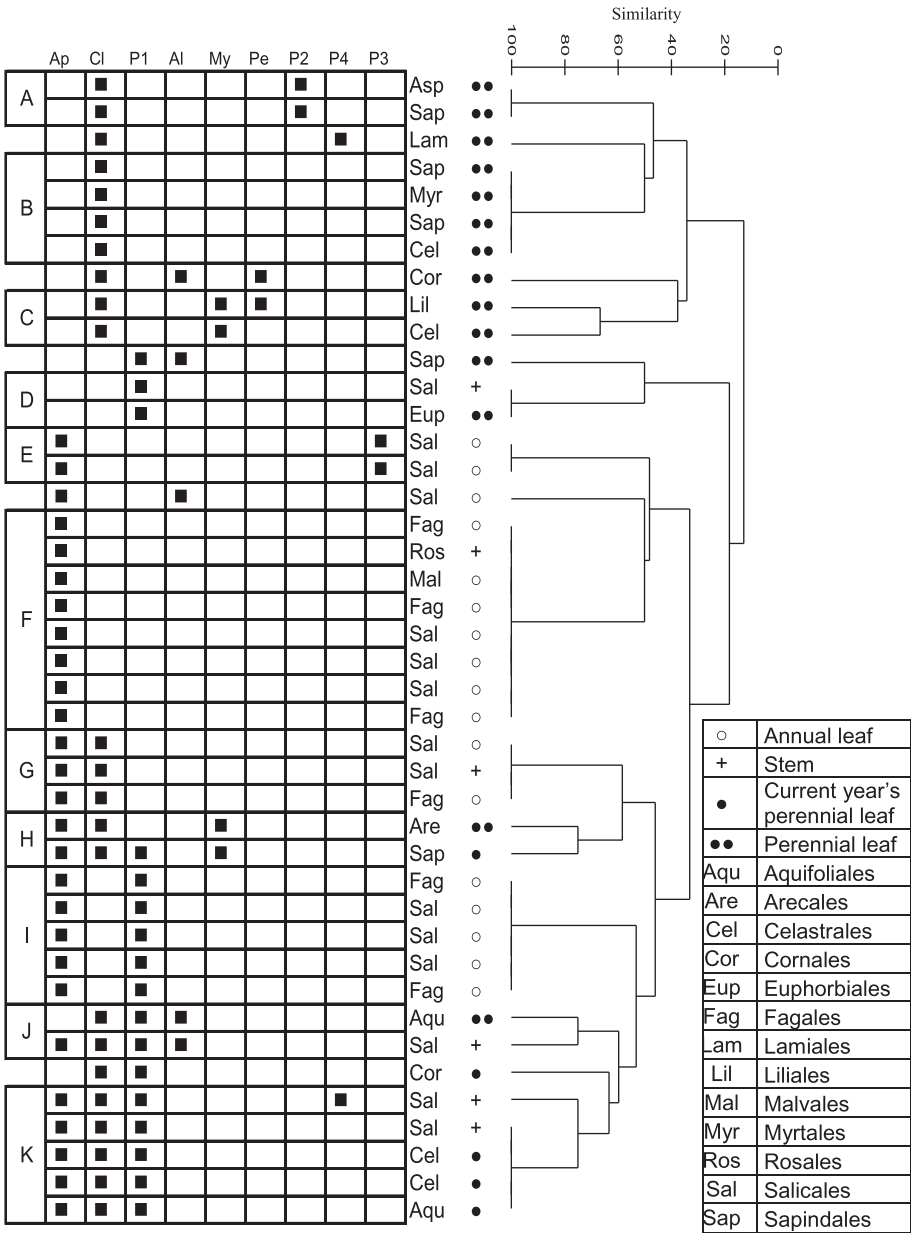

Fig 1 – Host plant characteristics mapped on a dendrogram reflecting the similarities in composition between the pigmented epiphytic fungal communities according to average linkage cluster analysis. The fungal groups are sorted from left to right according to their overall abundance: Al: *Alternaria*-1, Ap: *A. pullulans*-1, Cl: *C. cladosporioides*-1, My: Mycota-1, Pe: *Penicillium*-1, P1: *Phoma*-1, P2: *Phoma*-2, P3: *Phoma*-3, P4: *Phoma*-4. Clusters (A–K) resulted from truncating the dendrogram at a similarity of 65 %. The host plant order and sampled organs are also given.

greenhouse, while deciduous plants from different locations were studied. The following discussion is therefore based on the assumption that the 'host plant phenology' is the crucial factor and differences statistically ascribable to the 'host plant location' are also caused by the 'host plant phenology'. Nevertheless, this aspect is addressed again at the end of this paper.

The sooty patches were mostly caused by an association of taxa rather than by single fungal species. Reports on a heterogeneous species composition of sooty patches on leaves exist for a number of tropical crops such as *Citrus* (Perez *et al.* 2009) and *Mangifera indica* (Hamid & Jalaluddin 2006), while a single species causing sooty mould symptoms (*Capnodium salicinum*) was reported for *Salix* by Cannon (1999). While several pathogenic and endophytic fungi have been reported to be restricted to certain host plants, saprobic taxa, which dominate fungal communities, are thought to be less host-specific compared to pathogenic and endophytic fungi (Zhou 2001; Kodsueb *et al.* 2008). Our study did not reveal host selectivity of the fungi on host plant species level, but the host plant taxonomy at higher levels was reflected to some extent by the epifoliar fungal community composition. However, this

Table 1 – Differences between host plant genera, families and orders according to the composition of the pigmented epiphytic fungal communities. The *R* and *p* values are according to ANOSIM; insignificant results are not shown

	R	*p*
Plant genus (*n*)		
Salix (10), *Citrus* (3)	0.852	0.003
Salix (10), *Elaeodendron* (4)	0.547	0.006
Salix (10), *Coleonema* (2)	0.687	0.015
Salix (10), *Aucuba* (2)	0.915	0.015
Quercus (4), *Citrus* (3)	0.639	0.029
Salix (10), *Ilex* (2)	0.605	0.030
Plant family (*n*)		
Salicaceae (10), Rutaceae (5)	0.731	0.001
Salicaceae (10), Celastraceae (4)	0.547	0.013
Salicaceae (10), Garryaceae (2)	0.915	0.015
Salicaceae (10), Aquifoliaceae (2)	0.605	0.030
Fagaceae (4), Rutaceae (5)	0.444	0.048
Plant order (*n*)		
Salicales (10), Sapindales (5)	0.731	0.001
Salicales (10), Celastrales (4)	0.547	0.009
Salicales (10), Garryales (2)	0.915	0.015
Fagales (5), Sapindales (5)	0.580	0.016
Fagales (5), Celastrales (4)	0.494	0.016
Salicales (10), Aquifoliales (2)	0.605	0.030
Fagales (5), Aquifoliales (2)	0.682	0.048
Fagales (5), Garryales (2)	0.936	0.048

effect may have also resulted from interdependence between the factors 'host plant taxon' and 'leaf endurance'.

Since *A. pullulans*-1 occurred on 96 % of leaves younger than 1 yr but on only 20 % of the older leaves (Fig 3), the presence of *A. pullulans*-1 was regarded as a suitable indicator for sooty patches less than 1 yr of age. This assumption is in line with the finding of Woody *et al.* (2003), who showed that *A. pullulans* var. *pullulans* is the most common fungus on the deciduous leaf surfaces of *Malus domestica*. Analyses of fungal community succession in the phyllosphere of *Fagus crenata* (Osono 2002) revealed that *C. cladosporioides* and *Alternaria alternata* were present on young and senescent leaves, but the proportion of samples with *C. cladosporioides* increased over time, whereas samples with *A. pullulans* and *Phoma* sp. were more frequent on younger leaves. This indicates a succession from an *Aureobasidium* to a *Cladosporium*-predominated community and was statistically supported by the significant differences between the annual and perennial leaf colonizing pigmented epiphytic

Table 2 – Differences between the phenological groups according to the composition of the pigmented epiphytic fungal communities. The *R* and *p* values are according to ANOSIM. Significant differences between the groups are indicated in bold

Phenological groups	*R*	*p*
Stem, annual leaf	**0.308**	**0.005**
Stem, current year's perennial leaf	−0.040	0.686
Stem, perennial leaf	**0.287**	**0.022**
Annual leaf, current year's perennial leaf	**0.491**	**0.001**
Annual leaf, perennial leaf	**0.773**	**0.001**
Current year's perennial leaf, perennial leaf	0.116	0.144

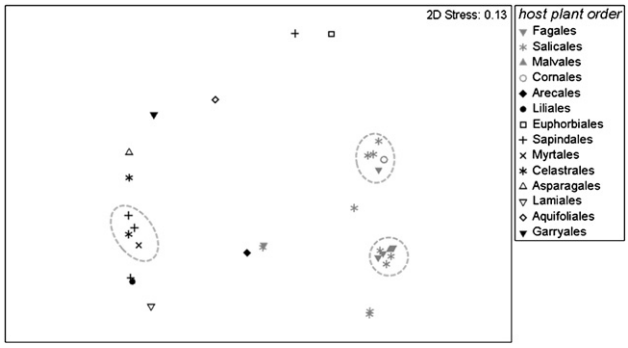

Fig 2 – Compositional similarities among the pigmented epiphytic fungal community visualized by non-metric multidimensional scaling (NMDS). Only the experimental groups with annual and perennial leaves were included in the analysis. Plant orders with annual leaves are coded in grey, and plant orders with perennial leaves in black. The encircled groups are zoomed in on by a factor of 4.

fungal communities ($p = 0.001$, $R = 0.773$) (the presence of *C. cladosporioides*-1 on the current year's perennial leaves is discussed further below). Accordingly, communities predominated by *C. cladosporioides*-1 seem to mark an advanced or climax stage on perennial tissues such as evergreen leaves, whereas the predominance of *A. pullulans*-1 indicates a rather 'ruderal' or 'unsaturated' developmental stage.

Accordingly, *A. pullulans*-1 successfully colonizes leaves in early developmental stages but it seems to be repressed by *C. cladosporioides*-1 over time. One possible reason for the early predominance of *A. pullulans* in the field may be its ability to produce antifungal substances such as aureobasidins (Takesako *et al.* 1991; McCormack *et al.* 1994). These are accumulated when nutrients are in short supply, most likely on the phylloplane. *Aureobasidium pullulans* also accumulates the polysaccharide pullulan when carbon and nitrate are in short supply (Dickinson 1986). The formation of such a pullulan layer is one mechanism known to prevail in the phylloplane and it is probably beneficial to initial colonizers.

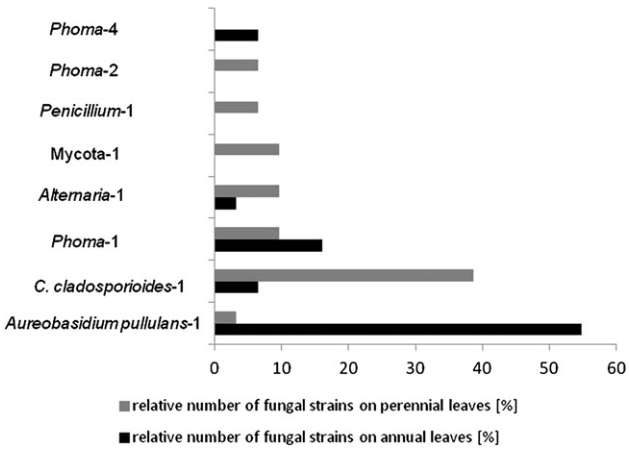

Fig 3 – Relative number of fungal isolates from annual and perennial leaf samples. The predominant fungal groups are shown.

Cladosporium, on the other hand, is not capable of forming a mucous layer. However, the reasons for the outperformance of *A. pullulans*-1 by *C. cladosporioides*-1 on older leaves remain speculative. A working hypothesis for future experimental studies may be that structural changes in leaf surfaces during the aging process affect microclimatic conditions such as wettability and temperature (Mechaber *et al.* 1996), and that the resulting microclimate of older leaves may be more favourable for *C. cladosporioides* than for *A. pullulans*.

In the annual phyllosphere habitat, succession of the fungal community is stopped at autumn by leaf fall. In the perennial phyllosphere habitat, succession can continue and later stages are predominated by *C. cladosporioides*. For all plants, however, fungal spores are the source of infection of newly developing leaves and the spectrum of airborne fungal spores actually includes all taxa that dominate the pigmented epiphytic fungal communities, i.e. species of the genera *Aureobasidium*, *Alternaria*, *Cladosporium* and *Phoma* (Ogulana 1974; Górny *et al.* 2002; Horner *et al.* 2004). The occurrence of *C. cladosporioides*-1 on the current year's perennial leaves could have resulted from spores released by *C. cladosporioides*-1 growing on older leaves in the immediate neighbourhood. Propagules of *C. cladosporioides*-1 are probably less common on deciduous trees because the sooty patches on stems were much less frequent than on perennial leaves. On the other hand, the regular presence of *C. cladosporioides*-1 in fungal communities that form sooty patches on the stems of plants with annual leaves shows that overwintering in the field may not account for differences between pigmented epiphytic fungal community compositions on annual and perennial leaves. Even though it is statistically not provable, the presence of *A. pullulans*-1 and *C. cladosporioides*-1 on evergreen plants in the greenhouse as well as on deciduous trees from different locations also renders an impact of the factor 'location' on composition of the fungal communities unlikely.

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Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.funeco.2011.12.005.

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Supplementary data: Details on name assignment, sequence analyses, and deposition of the isolated strains. Annotations of headings in the table: *$_1$ sequence accession number of the fungal strain (total sequences, number of best matching sequences obtained, numbers of outliers (i.e. sequences referring to taxa which are entirely different from the majority of the best matching sequences) numbers of ambiguities (i.e. sequences not determinable to a certain taxonomic unit), the minimal and maximal 'bit scores' their alignment obtained with the query sequence), *$_2$ voucher number of herbarised plant specimen.

Assignment	Assignment notes*$_1$	Herbarium accession numbers*$_2$
Aureobasidium pullulans-1	GU942836(100/48/10/42/922/970)	M-0128436
	GU942837(100/60/8/32/800/826)	M-0128422
	GU942844(100/52/13/35/905/948)	M-0128437
	GU942860(100/58/10/32/869/905)	M-0128460
	GU942862(100/58/10/35/883/922)	M-0128457
	GU942866(100/56/9/35/889/928)	M-0128459
	GU942867(100/57/10/33/883/922)	M-0128458
	GU942873(100/56/9/35/891/929)	M-0128454
	GU942875(100/61/11/28/819/841)	M-0128455
	GU942877(100/57/10/33/874/913)	M-0128452
	GU942880(100/58/10/32/876/915)	M-0128451
	GU942882(100/58/10/32/863/896)	M-0128450
	GU942883(100/70/10/20/815/837)	M-0128438
	GU942885(100/57/7/36/854/883)	M-0128432
	GU942887(100/55/12/33/874/913)	M-0128434
	GU942889(100/61/11/28/806/828)	M-0128441
	GU942895(100/63/9/28/808/824)	M-0128439
	GU942899(100/58/10/32/885/924)	M-0128453
	GU942901(100/56/10/34/880/924)	M-0128444
	GU942902(100/59/9/32/880/917)	M-0128443
	GU942910(100/57/11/32/878/917)	M-0128449
	GU942911(100/61/10/29/854/876)	M-0128448
	GU942923(100/55/9/36/896/907)	M-0128457
	CC18739 (–/–/–/–/–/–)§	M-0128456
	CC18730 (–/–/–/–/–/–)§	M-0128430
	CC18727 (–/–/–/–/–/–)§	M-0128428
	CC18733 (–/–/–/–/–/–)§	M-0128445

C. cladosporioides-1	GU942842(100/41/5/54/800/806)	M-0128430
	GU942847(100/47/6/47/835/841)	M-0128428
	GU942848(100/45/5/50/826/832)	M-0128471
	GU942849(100/47/6/47/752/758)	M-0128468
	GU942850(100/62/4/34/808/813)	M-0128467
	GU942851(100/55/12/33/859/863)	M-0128434
	GU942852(100/50/5/45/856/861)	M-0128432
	GU942853(100/48/5/47/809/815)	M-0128429
	GU942858(100/53/38/9/815/815)	M-0128438
	GU942859(100/53/38/8/797/797)	M-0128437
	GU942869(100/47/9/44/754/760)	M-0128456
	GU942892(100/60/6/34/817/819)	M-0128470
	GU942897(100/46/5/49/763/769)	M-0128431
	GU942907(100/82/5/13/736/780)	M-0128447
	GU942912(62/43/9/10/784/867)	M-0128448
	GU942914(100/57/6/37/830/861)	M-0128443
	GU942917(100/43/6/51/793/798)	M-0128445
	GU942921(100/55/11/34/867/867)	M-0128457
	CC18741(–/–/–/–/–/–)§	M-0128456
	CC18725(–/–/–/–/–/–)§	M-0128447
	CC18732(–/–/–/–/–/–)§	M-0128430
	CC18729(–/–/–/–/–/–)§	M-0128428
	CC18737(–/–/–/–/–/–)§	M-0128445
Phoma-1	GU942834(100/79/18/3/856/873)	M-0128435
	GU942835 (100/64/15/21/870893)	M-0128434
	GU942838(100/45/8/47/795/821)	M-0128440
	GU942839(100/66/12/22/869/891)	M-0128441
	GU942845(100/53/6/41/715/719)	M-0128438
	GU942856(100/62/21/17/828/845)	M-0128439
	GU942861(100/25/20/55/730/756)	M-0128460
	GU942871(100/22/2/76/767/802)	M-0128456
	GU942876(100/24/21/55/780/813)	M-0128453
	GU942881(100/24/22/54/789/833)	M-0128451
	GU942890(100/8/1/91/832/850)	M-0128465
	GU942920 (100/58/7/35/817/826)	M-0128457
	CC18742(–/–/–/–/–/–)§	M-0128456
	CC18726(–/–/–/–/–/–)§	M-0128447
	CC18736(–/–/–/–/–/–)§	M-0128430
	CC18728(–/–/–/–/–/–)§	M-0128428
	CC18738(–/–/–/–/–/–)§	M-0128445
Alternaria-1	GU942872(100/48/9/43/739/752)	M-0128456
	GU942891(100/38/7/55/843/854)	M-0128465
	GU942905(100/35/7/58/835/854)	M-0128449
	GU942906(100/42/9/49/791/802)	M-0128447
	GU942922(100/55/9/36/896/907)	M-0128457
Mycota-1	GU942840(1/1/0/1/761/761)	M-0128432
	GU942854(1/1/0/0/676/676)	M-0128431
	GU942896(1/1/0/0/719/719)	M-0128428
	CC18731(–/–/–/–/–/–)§	M-0128430
Phoma-2	GU942894(100/21/2/77/756/784)	M-0128468
	GU942857(100/21/2/77/815/843)	M-0128471
Phoma-3	GU942884(100/42/37/21/767/822)	M-0128438
	GU942886(100/73/13/14/560/610)	M-0128470
Phoma-4	GU942863(100/52/8/40/780/791)	M-0128457
	GU942916(100/35/9/56/846/856)	M-0128450
Penicillium-1	GU942841(100/97/3/0/765/832)	M-0128431

	GU942909(100/78/21/1/815/881)	M-0128447
Botryosphaeria-1	GU942865(34/25/8/1/481/510)	M-0128459
Coprinus xanthothrix-1	GU942879(2/2/0/0/1077/1107)	M-0128451
Cryptococcus flavescens-1	GU942868(53/27/11/15/632/701)	M-0128456
Dothideales-1	GU942864(22/6/1/15/806/894)	M-0128459
Mycota-2	GU942913(1/1/0/0/678/678)	M-0128449
Mycota-3	CC18749(–/–/–/–/–/–)§	M-0128456
Mycota-4	CC18724(–/–/–/–/–/–)§	M-0128447
Mycota-5	CC18735(–/–/–/–/–/–)§	M-0128445
Mycota-6	CC18734(–/–/–/–/–/–)§	M-0128445
Mucoraceae-1	GU942874(72/67/0/5/689/763)	M-0128454
Nectriaceae-1	GU942846(100/94/3/3/769/793)	M-0128439
Paraconiothyrium variabile-1	GU942915(13/11/2/0/935/965)	M-0128445
Pestalotiopsis-1	GU942843(100/81/2/17/985/1050)	M-0128429
Pleosporaceae-1	GU942878(89/55/0/34/852/946)	M-0128451
Umbelopsis ramanniana-1	GU942919(27/19/1/7/830/922)	M-0128457

Footnotes:

§ Assignment of strains was done by culture comparison with already determined taxa.

12.2 Manuscript 2

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Diversity of the *Capnocheirides rhododendri*-dominated fungal community in the phyllosphere of *Rhododendron ferrugineum* L.

Fabienne Flessa* and Gerhard Rambold

University of Bayreuth, Dept. of Mycology,

Universitätsstraße 30, D-95447 Bayreuth, Germany

*Corresponding author: fabienne.flessa@uni-bayreuth.de

Phone: +49 (0) 921 55-2455, fax: +49 (0) 921 55-2567

Short title: *Rhododendron* leaf-associated fungi

Abstract

Individuals of *Rhododendron ferrugineum* L. at natural sites within the mountain ranges and valleys Flüela, Julier, Monstein and Grimsel (in the cantons of Graubünden and Bern, Switzerland) were analysed to determine the occurrence of pigmented epifoliar fungi in their phyllosphere. Molecular data from the fungal isolates revealed a wide range of species to be present, forming a well characterized oligospecific community, with *Capnocheirides rhododendri* (Kunze) J.L.Crane & S.Hughes (Mycosphaerellaceae, Capnodiales, Ascomycota) being the most frequently occurring taxon. One group of fungi was exclusively isolated from the leaf surfaces and recognized as being residential epifoliar. A second ecological group was absolutely restricted to the inner leaf tissues and considered as truly endofoliar. Members of a third group occurring in both the epifoliar and endofoliar habitats were considered to have an intermediate life habit. Members of this latter group are likely to invade the inner leaf tissues from the outside after having established a mycelium on the leaf surface. Comparison of the degree of pigmentation between cultivated strains of the strictly epifoliar and strictly endofoliar community members provided some indication that epifoliar growth is to a certain degree correlated with the ability of the fungi to develop hyphal pigmentation. The endofoliar growth is assumed to entail a complete lack or presence of a more or less weak hyphal pigmentation.

Key words: Ascomycota, succession, epiphytic, endophytic, sooty moulds, alpine zone, dark pigmentation.

Introduction

Filamentose fungi with darkly pigmented hyphe in the vegetative part are typical colonizers of plant surfaces in various habitats of the world (Schoulties 1980). They are commonly referred to as 'sooty moulds' when found covering living leaves with a mat of more or less dense hyphal layers in the tropics (Kirk et al. 2008), or in a wider sense of all dark pigmented filamentose fungal taxa living on exudates of aphids and scale insects (Hughes 1976; Parbery & Brown 1986; Perez et al. 2009), on plant secretions (Weyman-Kaczmarkowa & Pedziwilk 2001), or on deposited pollen grains (Fokkema 1984). The circumscription of the group of sooty moulds has been sometimes restricted to certain taxonomic groups (Hughes 1976). Earlier studies on the composition of sooty patch-forming fungal communities on leaves of Central European deciduous and evergreen plants revealed a lack of any significant interdependence between the fungal taxonomic spectrum and the host plant taxa (Flessa et al. 2012), in contrast to what is known about various phytopathogenic fungal groups (O'Kane 1910; Hasan 1974; Hughes 1976; Goos 1978; Francis 2002). Recently, dependency on the life spans of colonized leaves has become apparent with regard to community composition (Flessa et al. 2012). Fungal taxa that are well known as epifoliar fungi (e.g. *Aureobasidium pullulans, Alternaria*, *Cladosporium* and *Phoma*) (Webb and Mundt 1978; Fenn et al. 1989; Yang et al. 2001; Osono 2002) are also reported as regular endophytic fungi (Suryanarayanan et al. 2005; Osono & Masuya 2012).

The present study is focused on the leaf-associated fungal communities of *Rhododendron ferrugineum* L. (Ericaceae). This plant is one of nine species of the genus with a natural occurrence in Europe (Crane et al. 2004) and is distributed from the European Alps to the Pyrenees (Ozenda 1985). This evergreen shrub is known regularly to host dark-pigmented, leaf surface-colonizing fungi (Corda 1829; Crane & Hughes 1982) and dominates several plant communities in the northern European Alps at altitudes from 1600 to 2200 m (Escaravage et al. 1998). *Rh. ferrugineum* is characterized by rather conspicuous glands on the lower leaf surface (Kratzmann 1910), the exudates of which may provide a nutritive source for various kinds of microbial organisms.

Sooty mould symptoms on the lower leaf surfaces of *Rh. ferrugineum* and *Rh. hirsutum* L. were first described by Kunze in Corda (1829) for the species *Torula rhododendri* Kunze. The taxon was subsequently recognized as not being congeneric with the generic type of *Torula* Pers., *T. herbarum* (Pers.) Link, and was transferred into the monospecific genus, *Capnocheirides* J.L.Crane & S.Hughes (Crane & Hughes 1982). Given their status as among the most popular ornamental plants, cultivars of *Rhododendron* were objects of quite a number of extensive studies concerning morphology and ecology of their leaf pathogens, such as the rust fungus *Caeoma tsukubaens* (Crane et al. 2004) and members of the genus *Chrysomyxa* (Hiratsuka & Sato 1969; Crane 2001; Nierhaus-Wunderwald 2002). However, there is still a lack of detailed studies with a focus on the community composition of *Rhododendron* leaf-associated, non-phytopathogenic fungi.

In the present study, *Rh. ferrugineum* leaf-colonizing epiphytic and endophytic fungi were analysed to determine the community composition and possible life strategies of their members. Furthermore we addressed how far there was an overlap between the compositions of fungal communities found in and on the leaves, based on the assumption that several of the mostly pigmented epifoliar taxa are likely to invade the leaf interior from the outer surface during leaf development. Such behaviour of direct ingression into the leaves has been discussed in detail for endophytic fungi of *Viscum album* ssp. *austriacum* (Wiesb.) Vollm. and associated ascomycetes by Peršoh et al. (2010). The opposite behaviour observed in primarily endophytic fungi, where epifoliar growth takes place at later stages of development (e.g. Tanaka 2010). It is also to be expected that there are certain differences in morphological traits between the *Rh. ferrugineum*-colonizing fungal groups with an obligately epifoliar and obligately endofoliar life habit such as, for instance, the intensity of hyphal pigmentation in response to different degrees of UV radiation (Rangel et al. 2006).

The objective of the present study was to test the following hypotheses: A) *Rh. ferrugineum* leaf-associated fungal communities represent multipartite associations; B) epifoliar pigmented fungi on *Rh. ferrugineum* build up an oligospecific community that is different in composition from fungal sooty mould associations colonizing other plants at lower altitudes in Central Europe; C) differences in *Rh. ferrugineum*-associated fungal leaf community compositions in their natural habitats are mainly due to age of the host leaves and the altitudinal vegetation zones, respectively; and D) parts of the endofoliar fungal community in *Rh. ferrugineum* pertain to primarily epifoliar (pigmented) fungi and access their habitat by invasion from the plant surface.

Material and Methods

Collections

In order to screen for epifoliar fungal taxa, host plant individuals of *Rh. ferrugineum* were sampled in August 2007 at four separate natural sites in the Alps of Switzerland: Grimsel Valley, Flüela Mountain Pass, Julier Mountain Pass, and the surroundings of Monstein. To screen for endofoliar taxa, additional host plant samples from three sites were also collected in August 2007 (Julier and Monstein surroundings) and in August 2008 (Flüela). All host plants occurred in open populations, as defined by Pornon et al. (1996), having a coverage of = 25% of the area. In each geographic area samples were taken at the following three altitudinal vegetation zones. 1) European larch forest zone with *Larix decidua* Mill. at 1770–1980 m alt. In the region of the Julier mountain pass, *Rh. ferrugineum* was lacking in this forest type and individuals in the Albula Valley, parallel to Julier Valley, were sampled instead. 2) Mountain pine zone with *Pinus mugo* Turra at 2000–2200 m alt. 3) Alpine zone at 2100–2300 m alt.

Switzerland, Bern, Grimsel Valley: **G1.** 46°33'43.11" N, 8°20'6.45" E, 2150 m alt. *Vaccinium myrtillus* L. shrubs in open situation (M-0126011). **G2.** 46°34'23.64" N, 8°20'11.00" E, 1870 m alt. (M-0126006; M-0126007). **G3.** 46°35'32.59" N, 8°19'32.62" E, 1750 m alt. Slope with dominant *Pinus mugo* (M-0126009; M-0126010). **G4.** 46°35'48.50" N, 8°19'33.55" E, alt. 1620 m. Slope behind small creek with *Larix decidua* (M-0126004; M-0126005). ***Graubünden, Monstein* surroundings**: **M1.** 46°40'57.96" N, 9°45'57.29" E, 2150 m alt. Alpine grassland with dominant *Vaccinium myrtillus* (M-0125984, M-0125985). **M2.** Alp Mäschenboden. 46°41'5.31" N, 9°47'2.32" E, 2090 m alt. Alpine grassland with dominant *Vaccinium myrtillus* (M-0125990, M-0125991). **M3.** 46°41'23.67" N, 9°47'7.35" E, 1980 m alt. Slope with dominant *Larix decidua* in the surroundings (M-0126001). ***Graubünden, Bever, Julier Mountain Pass***: **J1.** 46°27'43.42" N, 9°40'54.73" E, 1850 m alt. Farmed grassland, without *Larix* (M-0125982; M-0126003). **J2.** 46°27'54.51" N, 9°42'28.96" E, 2080 m alt. Predominant *Pinus mugo*, no *Larix* (M-0125993, M-0125992). **J3.** 46°28'16.18" N, 9°43'19.1" E, 2233 m alt. *Vaccinium myrtillus* shrubs, without *Pinus mugo* and *Larix* (M-0125998, M-0125999). ***Graubünden, Flüela Mountain Pass***: **F1.** 46°44'46.48" N, 9°57'18.55" E, 2300 m alt. Slope with *Vaccinium myrtillus* in open situation (2007: M-0126020, M-0126021; 2008: M-0126014, M-0126015). **F2.** 46°44'34.41" N, 9°58'32.45" E. 2200 m alt. Slope with dominant *Pinus mugo* (2007: M-0126016, M-0126017; 2008: M-0125977, M-0125976). **F3.** 46°44'40.24" N, 9°59'01.20" E, 2000 m alt. Slope with predominant *Pinus mugo* (2007: M-0126018; 2008: M-0125981). **F4.** 46°45'03.49" N, 10°02'57.59" E, 1770 m alt. with dominant *Larix europaea* (2007: M-0126019; 2008: M-0125983). ***Graubünden, Palpuogna***: **P1.** 46°34'52.92" N, 9°47'03.03" E, 1920 m alt., with dominant *L. europaea* (M-0126012, M-0126013).

Isolation and cultivation of the fungi

Two leaved twigs per *Rh. ferrugineum* individual and collecting site were sampled. Leaves covered by macroscopically recognizable dark pigmented mycelia were collected twice per plant individual at the same time, and fungi were isolated from three segments of the mycelium of two different leaves from the current year (cyl, 5–6 months) and previous year (pyl, 17–18 months). The plant material was stored at < 10 °C and was processed immediately after being transported to the laboratory. Leaf glands of *Rh. ferrugineum* are restricted to lower leaf surfaces, and do not occur on the upper leaf surfaces. In order to obtain epifoliar fungi, the mycelium was dissected from the leaf and washed in sterile tap water to remove the majority of the adhering airborne fungal spore material. For isolation of endofoliar fungi, the plant parts were surface-sterilized in 70% ethanol for 1 min, in 1.2% sodium hypochlorite for 3 min, rinsed three times in sterile water for 1 min, and subsequently wrapped in sterile paper towels for about 15 min to remove water from the surface (Peršoh et al. 2010). Disc-shaped segments (20 mm^2) were cut from the leaf centre of two surface-sterilized leaves per maturity stage, twig and plant individual. Three discs were cut from each leaf, one from the basal end, one from the middle and one from the tip of the leaf. In order to obtain individual fungal strains, the pieces of washed mycelium (epifoliar fungi)

and the punched discs (endofoliar fungi) were subsequently transferred to Petri dishes of 5.5 cm diam. containing yeast-malt medium (4 g glucose, 10 g malt extract, 4 g yeast extract, and 12 g agar per litre) with 0.1% tetracycline to suppress bacterial growth. The Petri dishes were incubated at 15 °C and observed daily to record emergence of hyphae. Emerging colonies were separated and transferred onto new plates. One fungal strain of each operational taxonomic unit (OTU), with at least three isolates in total, and an assortment of OTUs with two isolates and singletons were deposited in the collection of the Jena Microbial Research Collection (JMRC). Accession numbers are listed in Appendix A. The OTUs (n = 3) were examined and compared for consistency with morpho-anatomical concepts using a light microscope.

Pigmentation tests

The fungal strains were classified based on hyphal pigmentation using cultures grown on three different growth media: yeast-malt medium (4 g glucose, 10 g malt extract, 4 g yeast extract, and 12 g agar per litre), carrot-agar (4 ml carrot juice, 12 g agar per litre) and *Rhododendron*-agar (20.75 g crushed fresh leaves from *Rh. ferrugineum*, 12 g agar per litre, with fresh twigs cut in the field, and the cut surfaces wrapped in a damp towel and kept in a plastic bag, stored at 5 °C until usage). The Petri dishes were incubated at 15 °C and fungal cultures were examined after one month for macroscopically recognizable pigmentation. In order to demonstrate coherence between obligately endofoliar life habit and pigmentation, the hyphal pigmentation was tested for mycelia on yeast-malt-agar, carrot-agar and *Rhododendron*-agar *Rh.*. One piece of every dark coloured mycelium was therefore placed in tubes with acetone or methanol. Pigmentation not soluble in either solvent was considered to be hyphal pigmentation. CMYK-values for pigmentation classified as ‘dark’ were: C: 39–73%; M: 50–75%; Y: 51–94%; K: 21–83%. CMYK-values for pigmentation classified as ‘light’ or ‘lacking’ were: C: 28–49%; M: 32–48%; Y: 33–61%; K: 0–10%.

DNA extraction, amplification, sequencing of the ITS nrDNA and grouping of the ITS nrDNA

Pure cultures of the fungal isolates were preselected according to the following phenotypic traits: presence/absence and type of aerial mycelia, growth form, and type of pigmentation. For every leaf sample, representatives of each morphotype were chosen for sequencing. Consistency between molecular name assignments and the morpho-anatomical concepts was confirmed for multiton taxa using light microscopic examination (Peršoh et al. 2010; Flessa et al. 2012).

The Charge Switch® gDNA Plant Kit (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA) was used to isolate DNA from the culture material. Cell disruption was accomplished using the Fast Prep FP120 (Bio101, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) at a speed of

6.0 ms^{1} for 2 × 40 s. Polymerase chain reaction (PCR) was performed using a MWG Biotech Primus 96 plus thermocycler. Double-stranded sequences of the ITS rRNA gene were obtained and further processed according to the method of Triebel et al. (2005). Sequences were deposited in the NCBI GenBank under accession numbers as listed in detail in the table in Appendix A.

For statistical analysis, fungal isolates were grouped according to their ITS rDNA sequence similarities as described by Peršoh et al. (2010). Briefly, pairwise similarities among length-adjusted sequences were calculated using the BLAST application 'blastall' (v. 2.2.18). The resulting tabular output dataset was transformed using the function 'simMatrix', package 'RFLPtools' (Flessa et al. 2010) in R (R Development Core Team 2010). A cluster analysis was conducted using the function 'hclust' with the clustering method 'average linkage' in R, and clusters with minimal similarities of 96% were grouped into OTUs (Flessa et al. 2012).

Sequence-based assignment of names to similarity groups

Taxon names were assigned to the clusters to allow comparison with the results of other studies, but all data were exclusively analysed on the basis of sequence clusters, independent of the assigned names. The nomenclature followed the Index Fungorum (http://www.indexfungorum.org) and the higher classification MYCONET (http://www.fieldmuseum.org/myconet).

Names were assigned to the sequences, and correspondingly to the isolates, based on the nearest relatives determined by the 'Mega BLAST' (Zhang et al. 2000) results in the NCBI database (http://www.ncbi.nlm.nih.gov; status: January 2010). A consensus taxonomic assignment was compiled from the names of deposited sequences with a 'bitscore' of at least 90% of the best matching sequences, following the approach of Peršoh et al. (2010). Details on the name assignment are included in the supplementary data (Table 1). Groups, i.e. clusters, were named according to the sequences they included, with consecutive numbering of groups that would otherwise have identical names. An analysis of similarity (ANOSIM; as defined by Chapman & Underwood 1999) served further to assess the reliability of the assigned names by analysing the sequence similarity matrix (see above) against the classification linked to the assigned names.

Separate BLAST searches for each group with n = 5 in GenBank were conducted to obtain all sequences sharing at least 90% for any query sequence of each group. Similarity matrices were calculated as described above, and imported into Primer 6 (Plymouth Routines, v. 6.1.6) in order to conduct non-metric multidimensional scaling (NMDS) analyses. Following Peršoh & Rambold (2011), substrate data of all isolated strains, including published reference data, were mapped onto the NMDS graphs (Fig. 1 Table 1). The assigned taxon names, as well as reliability estimates of the sequence-based name assignments, are provided in Appendix A.

Data analysis of fungal community trends

The binary matrix coding for the presence or absence of the fungi of each cluster in each sample was transformed into a similarity matrix, based on Jaccard distances using PRIMER 6 (Plymouth Routines, v. 6.1.6). The occurrence of fungal OTUs (singletons were excluded) was noted for the samples of each host plant. The respective isolation source, i.e. current year's leaves ('cyl', i. e. 5–6 months old) or previous year's leaves ('pyl', i. e. 17–18 months old), the geographical location and also the altitudinal vegetation zone, were each coded as grouping factors. Analysis of similarity (ANOSIM) was conducted to assess the impact of each factor on the sample grouping. ANOSIM calculates the p-value, which is considered to indicate significant differences when below 0.05. The R-value was used to indicate to what degree the respective factors explained groupings among samples. R-values >0.75 were interpreted as indicating clearly separate groups, $R > 0.5$ overlapping, but clearly different, and $R < 0.25$ barely separate groups (Chapman & Underwood 1999). The similarity of epiphytic fungi forming sooty patches and endophytic fungal assemblages was compared using the Sørensen Similarity Index (QS) as follows: $QS = 2a/(2a + b + c)$, where a is the number of OTUs occurring in both communities, while b and c are the numbers of OTUs exclusively epiphytic and endophytic fungi, respectively (Osono & Mori 2004; Kharwar et al. 2010).

Results

Grouping of the isolates and assignment of taxon names

In total, 323 sequences were gained from 153 epifoliar and 106 endophytic fungal isolates, originating from the study sites together with sequences from an additional 64 isolates of epifoliar fungi from the Grimsel region. By using cluster analysis, 253 of the 323 sequences were grouped into 111 clusters and 70 singletons. ANOSIM revealed the factor 'taxonomy', i.e. name assignment according to the BLAST search results, in order to explain the significance ($p < 0.05$) of genetic dissimilarities among the sequences at the various taxonomic levels, i.e. at species ($p = 0.01$, $R = 1$, $n = 119$), genus ($p = 0.01$, $R = 1$, $n = 240$), order ($p = 0.01$, $R = 0.978$, $n = 231$), family ($p = 0.01$, $R = 1$, $n = 140$), and subclass ($p = 0.01$, $R = 0.811$, $n = 241$) levels. Comprising 287 sequences, OTUs assigned to Ascomycota were the predominant group, whereas only nine OTUs were assignable to Basidiomycota, and four OTUs to Zygomycota. Among the Ascomycota, the most abundant groups represented Dothideomycetes with 167 OTUs (133 epifoliar, 34 endofoliar), followed by Leotiomycetes with 53 OTUs (30 epifoliar, 23 endofoliar), Eurotiomycetes with 38 OTUs (22 epifoliar, 16 endofoliar), Sordariomycetes with 19 OTUs (nine epifoliar, 10 endofoliar), and 10 OTUs (four epifoliar, six endofoliar) of unknown relationships. The distribution of fungal genotypes ($n = 3$) according to their epifoliar and endofoliar occurrences are displayed in Fig.2.

Sequence similarities between groups of *Rh. ferrugineum*-associated fungal strains and all published sequences showing at least 90% similarity to the respective group revealed the following results: Published sequences with a high similarity to the group of OTUs comprising *Sarcinomyces*-1 and *Capnocheirides rhododendri*-1 (Fig. 1, graphs **A**, **K**) originated from heterogeneous substrate types. Clusters comprising *Cladosporium*-1 to -4 (**E**–**H**) corresponded to sequences of OTUs derived from water samples and surface-sterilized or non-surface-sterilized living plant material. Sequences of the *Penicillium*-1 cluster (**M**) mainly corresponded to sequences of OTUs isolated from surface-sterilized living plant tissues and air samples. Results for smaller OTUs are displayed in Fig. 1. This analysis also revealed that the best matching sequences corresponded to those of surface-sterilized or non-surface-sterilized living plant organs, or leaf litter, and, to a much lower degree, to sequences from plant root and soil-derived OTUs.

Observations on the life habit of *Capnocheirides rhododendri*

Capnocheirides rhododendri-1 was predominant on the current and previous year's leaves (82.9% and 50%, respectively). The decrease in the extent to which it was found on the previous year's leaves may be correlated with observations about host plant leaves. Sooty patches due to *C. rhododendri* occurred on the lower surface of *Rh. ferrugineum* leaves. On the current year's lower leaf surfaces the fungus always formed a considerable number of small and dispersed sooty patches. Their distribution is likely to have coincided with leaf glands. These glands were observed to start as green, and covering the leaf surface of the current year's leaves rather densely, but protruding and turning to brown on the previous year's leaves. The gland activity was observed on leaves from fresh twigs which were cut in the field and stored at 5 °C until use. On the lower leaf surfaces of the current year's leaves, drops of a viscous, yellowish brown secretion were detected, whereas the glands of the previous year's leaves were non-functional and remained dry. Sooty patches caused by *C. rhododendri*-1 on the previous year's leaves were observed to fuse together, forming a contiguous layer more or less covering the whole lower leaf surface. Observations on *C. rhododendri*-1 in culture showed that growth is rather slow on all tested media when compared, for example, to *Cladosporium* spp.

Alpha-diversity of epifoliar fungi

In the present study, 93 visibly infected leaves (cyl/pyl) were investigated. Sooty patches were only observed on the lower leaf surface, correlated to occurrence of leaf glands in the host plant, these also being restricted to lower leaf surfaces. Samples with only one OTU were obtained from 35 leaves (30 cyl, 5 pyl). However, oligospecific communities mainly occurred: two OTUs were present on 24 leaves (cyl/pyl: 7/17), three on 16 (cyl/pyl: 4/12), four on 10 (cyl/pyl: 0/10), five taxa on five (pyl), and communities with six to eight OTUs in one sample (pyl). As result of the ANOSIM, differences in fungal

community compositions of parallel samples from corresponding plants from the same sampling sites could be ignored.

According to the ANOSIM analysis, the factor 'leaf age' was significant (p = 0.008) but the rather low *R* value (*R* = 0.068) indicated that the leaf age groups largely overlap. No fungal OTU was found to occur on all leaves. Aside from the predominant *C. rhododendri*-1 (82.9% cyl, 50% pyl), the fungal OTUs with overall frequencies > 10% were *Cladosporium*-3 and *Sarcinomyces*-1. While *C. rhododendri*-1 was more frequent on cyl, the frequency of *Cladosporium*-3 was higher on pyl, and *Sarcinomyces*-1 exhibited similar frequencies on leaves of both stages. Among the OTUs found in more than 5% of the samples, Leotiomycetidae-1 and *A. pullulans*-1 preferentially occurred on older leaves, whereas *Cladosporium*-4 and *D. pityophila*-1 had a balanced distribution between both leaf stages (Table 2). The factor 'sampling site' was significant (p = 0.009), but the very low *R*-value (*R* = 0.084) indicated that the groups largely overlapped according to sampling site.

Alpha-diversity of endofoliar fungi

In 56% of cyl and pyl leaves, fungal occurrence was observed, with 25% in the cyl, and 88% in the pyl. In 14 leaves (four in cyl, 10 in pyl) one single fungal species was detected. Oligospecific communities were most commonly observed: two species were present in 16 leaves (five cyl, 11 pyl), three species in seven leaves (one cyl, six pyl), four species in three leaves (pyl), five species in three leaves (pyl), and six species were present in two leaves (pyl).

The factor 'leaf age' was not significant (*p* = 0.137, *R* = 0.045). Predominant fungal OTUs, colonizing > 10% of the cyl or pyl (> 10% of the leaves), were *Cladosporium*-1 and 2, *Hypoderma rubi*-1, *Penicillium*-1, *Sarcinomyces*-1 and Vibrisseaceae-1. More than one third of the cyl leaves was inhabited by *Penicillium*-1, showing lower frequencies on old leaves. *Cladosporium*-4, Ascomycota-1, *Cladosporium*-3 and *Preussia*-1 were less common, but showed a similar trend. *Sarcinomyces*-1 and *Cladosporium*-2 exhibited an opposite tendency. Fungi occurring in > 10% of pyl and lacking in cyl were *Cladosporium*-1, Dothideomycetes-1, *Hypoderma rubi*-1 and Vibrisseaceae-1. Although most members of the endofoliar fungal community were not significantly influenced by leaf age, *Penicillium*-1 and Ascomycota-1 tended to prefer the younger leaves. Global ANOSIM was significant for the factor 'geographical region' (*p* = 0.001, *R* = 0.131), and for the factor 'sampling sites' over all geographical regions (*p* = 0.001, *R* = 0.248).

The effect of altitudinal zonation

OTUs either occurred in all the three altitudinal vegetation zones (AVZ), in two adjacent AVZs, or were restricted to a single zone (Fig. 3) (zones are characterized in the Material and Methods). OTUs occurring in all the three AVZs apparently formed a community with obligately associated fungal species on

Rh. ferrugineum. Such a community contains *A. pullulans*-1, *C. rhododendri*-1, *Cladosporium*-2, *Cladosporium*-3, *Cladosporium*-4, *D. pityophila*-1, *H. juniperi*-1, *Leotiomycetidae*-1, *Penicillium*-1, *Phialocephala*-1, and *Sarcinomyces*-1. Records from two adjacent AVZs exist for *Hypoderma rubi*-1, Vibrisseaceae-1, Mycota-2 and *Preussia*-1 for the two upper zones (*Pinus* and alpine zones), and *Cladosporium*-1 and *S. conigenus*-1 were found in the two lower zones (*Larix* and *Pinus* zones). While none of the fungal OTUs was restricted to the alpine zone, Dothideomycetes-1 exclusively occurred in the *Larix* zone, and Ascomycota-1 and *Sydowia*-1 were only recorded from the *Pinus* zone.

AVZs had no significant effect on the composition of epifoliar fungi ($p = 0.578$, $R = –0.009$), and had only a minimal effect on the endofoliar one ($p = 0.016$, $R = 0.063$). Significant ($p = 0.018$) but slight ($R = 0.112$) differences were found between the *Larix* and alpine zones, the *Pinus* and *Larix* zones ($p = 0.02$, $R = 0.119$), and between the *Larix* and *Pinus* zones ($p = 0.043$, $R = 0.083$). Differences in fungal community compositions between the *Pinus* and the alpine zone were insignificant ($p = 0.19$, $R = 0.024$).

Examination of the epiphytic and endophytic fungal communities

The parallel examination of the communities of epiphytic and endophytic fungi on *Rh. ferrugineum* leaves was motivated by the hypothesis that some of the mostly pigmented phyllosphere fungi may have different life strategies. A considerable number of OTUs was restricted to the leaf surface (Amphisphaeriaceae-1, Ascomycota-3, *A. pullulans*-1, Dothioraceae-1, *Hypocrea*-1, Leotiomycetidae-2, *Phoma*-1, Pleosporales-1 and 2, *S. conigenus*-1, *Phaeosphaeria*-2 and *Sydowia*-1) and are therefore considered to have an exclusively epifoliar life strategy. A similar number of OTUs was found to have a strictly endofoliar occurrence (Ascomycota-1, *Cladosporium*-2, *C. empetri*-1, *Geomyces*-1, *H. rubi*-1, Mycota-3, *Physalospora*-1 and -2, *Preussia*-1 and -2 and *U. ramanniana*-1). A third group of OTUs occurred in both habitats (*Botryotinia*-1, *C. rhododendri*-1, Capnodiales-1, *Cladosporium-1,* -3 and -4, *D. pityophila*-1, Dothideomycetes-1, *H. juniperi*-1, Hyaloscyphaceae-1, Leotiomycetidae-1, *L. infectoria*-1, Mycota-2 and -4, *Penicillium*-1, *Phialocephala*-1, *Sarcinomyces*-1 and Vibrisseaceae-1). The Sørensen Index of Similarity gave a result of 0.83 (n = 3). The Sørensen Index of Similarity enables comparisons with the data of other studies regarding to the common sooty moulds and endophytic fungi. It is indicated that epiphytic and endofoliar fungal communities overlap in their species spectra.

Pigmentation was considered to be dark if it was not soluble in solvents, and CMYK-values for pigmentation were detected. Fungi exhibiting brown pigmented cells on all three culture media were considered as obligately pigmented taxa, while those pigmented on just one or two culture media were considered to facultatively pigmented taxa. Strains lacking any pigments on all three culture media were considered as indicators for the inability to produce any dark pigments. Two epifoliar fungi (*Phoma*-1 and *Sydowia*-1) were recognized to be obligately pigmented, and all others belonged to the facultative pigmented group (Table 3). Additionally, *C. rhododendri*-1, which has been considered as only an occasional invader, was found to be ‘obligately pigmented’. Among the endofoliar taxa, only three were obligately

pigmented (Ascomycota-1, *Cladosporium*-2, and *U. ramanniana*-1), but two were completely lacking pigmentation (*Physalospora*-1 and *Preussia*-1). The remaining taxa belonged to the group of facultatively pigmented group. *Penicillium*-1, which has been considered to occur occasionally on leaf surfaces, also lacked any pigmentation. When considering all OTUs occurring in both habitats, the obligate pigmentation type in culture was the most frequently found.

Discussion

Methodology

The observed colonization frequency (56%) of the leaves by endofoliar fungi in this study is within the range of comparable cultivation-based studies (Fisher et al. 1994; Arnold & Lutzoni 2007). Despite only a fraction of microbial populations being assessed by culture-based methods (Yang et al. 2001), this method was evidently sufficient for detecting the majority of fungal strains causing symptoms of sooty mould, because even *C. rhododendri*, a slowly growing fungus with a very narrow host spectrum and occurring on the leaves of *Rh. ferrugineum* (Kunze in Corda 1829; Crane & Hughes 1982; Hughes 2007), could be regularly recognized. Common taxa described in other studies of dark pigmented epifoliar communities, i.e. *A. pullulans*, *Chaetomium* sp., *Cladosporium* spp., *Lewia* sp., and *Phoma* spp. (Webb & Mundt 1978; Fenn et al. 1989; Yang et al. 2001; Osono & Takeda 2002), as well as taxa such as *Penicillium* spp. and *H. rubi*, common on plant genera other than *Rhododendron* (Hou et al. 2007; Egorova et al. 2008), were also detected. Therefore, cultivation-based studies can be considered to be adequate for observing potential compositional shifts in communities of isolated and presumably most of the characteristic taxa. Cultivation was also a precondition for testing the capability of the isolates to develop hyphal wall pigmentation, an assumed indicator for their natural life habit, as discussed below. Due to possible multi-factorial environmental impacts on the fungal community composition, re-inoculation experiments in a glasshouse with the isolated fungal strains were considered unfeasible and therefore not undertaken.

Observations on *Capnocheirides rhododendri*

In the context of community changes, *C. rhododendri* (being present in >80% of the cyl) clearly plays an important role in establishing initial fungal sooty patch communities. The observation that this taxon only grew on or in close vicinity to the leaf glands of the host plant indicated that growth and predominance of *C. rhododendri* on the lower leaf surface is favoured by its effective use of leave gland secretions, these being of high nutritive value for the fungus. Evidence of this assumption is deduced from the observation that not only contiguous fungal layers were formed to some degree on the leaf surface, such as by pigmented epifoliar fungi at lower altitudes (Flessa et al. 2012), but also considerable

numbers of incoherent, small sooty patches. This is also supported by the observation that the appearance and activity of the glands changed dramatically during ageing of the leaves. Once they turned brown and protruded on the previous year's leaves, possibly even stopping their secretion, so this nutritional source for *C. rhododendri* was no longer available and the fungus lost its advantage over other epifoliar, less specialized fungi. As its speed of growth is relatively low it was soon outperformed and exhibited a dramatic decrease to an occurrence of only 50% on the pyl.

Alpha-diversity and changes in community structure in relation to the geographic distribution and altitudinal range of the host plant.

The 323 fungal isolates gained from the host plant were assigned to 181 OTUs, which indicated that *Rh. ferrugineum* hosts a broad variety of endofoliar and epifoliar fungal species.

C. rhododendri, which was present on >80% of cyl, appeared unable to suppress the growth of other epifoliar fungal groups, because the sooty patch symptoms were mostly caused by an association of various fungal taxa (58 samples) rather than by only one species (30 samples).

In the cyl, the frequency of occurrence of oligospecific endofoliar communities was considerable, but was still higher on the pyl. This indicated that, even when colonization frequency is relatively low, once an endofoliar fungus has colonized the leaf, it is followed by additional fungi and an oligospecific community is established.

In sooty patches on pyl, *C. rhododendri*-1 was less frequent (50% of pyl samples) than on cyl. However, in contrast to findings in other dark pigmented epifoliar fungal communities (Flessa et al. 2012), there was no transition towards a community being dominated by another fungal species. Only in four samples was a transition to a more complex, i.e. oligo-specific aggregate observed with a tendency towards co-dominance with *Cladosporium*-3 on pyl.

Some of the isolated epifoliar strains appeared to belong to ubiquitous taxa, and several others to obligately alpine ones. Taxa from the genus *Sarcinomyces* were, for instance, also found on marble in the Mediterranean region (Wollenzien et al. 1997; Sert et al. 2007). *A. pullulans* and *Cladosporium* sp. are very common fungal taxa on plants in habitats of lower altitudes (Flessa et al. 2012). *S. conigenus* was found on sugar maple and white oak leaf samples from streams (Das et al. 2006). Besides these ubiquitous taxa, obligately alpine sooty mould symptom-causing OTUs could also be recognized (*C. rhododendri*-1, *H. juniperi*-1 and *Sydowia*-1). Analysis of published sequences showing 90% similarity to OTUs in this study indicated that Vibrisseaceae-1 may represent an alpine fungus that is exclusively associated with *Rh. ferrugineum*. The most frequent fungal taxa (*Penicillium* sp. and *H. rubi*) do not exclusively occur in alpine habitats. *Penicillium* spp. are common in *Rhododendron* (Egorova et al. 2008), and also in other Ericaceae (Stohr & Dighton 2004), and are assumed to inhibit pathogens (Nix-Stohr et al. 2008). *H. rubi* is also a typical fungus colonizing *Rhododendron* (Hou et al. 2007).

Surprisingly, composition of epiphytic fungi was not affected by the altitudinal vegetation zones, the most abundant groups being isolated from all three zones. Therefore, we assume that the community of the epiphytic, sooty patch-forming fungi on *Rh. ferrugineum* are not influenced by fungal taxa derived from the surrounding vegetation. In contrast, significant differences among the *Larix*, *Pinus* and alpine vegetation zones existed with regard to the community composition of endophytically growing fungi. These differences with respect to the predominant surrounding vegetation indicated that there may be a direct or indirect exchange of leaf-inhabiting fungi between *Rh. ferrugineum* and certain other plant species typical of the respective vegetation zone. In the absence of studies of leaf samples from *Larix* and *Pinus* and other plant species of montane to alpine habitats, we were unable to verify the assumption of a possible horizontal distribution of certain endophytic strains. Another possibility is the existence of differences in microclimate (e.g. temperature and precipitation) between the three zones, which may be influenced by the vegetation (i.e. alpine zone has the lowest neighbouring plants, the *Pinus* zone has an intermediate height of plants compared with the *Larix* zone, which exhibits the highest plants of the three vegetation zones), and also abiotic factors.

The 'leaf age' factor show no or minimal effect on the composition of the endofoliar fungi. The significant shifts of epifoliar fungal communities between cyl and pyl were probably due to the changing availability of certain cell compounds in the host plant. Studies on cell compound shifts correlated with leaf age were undertaken in earlier studies using the leaves of *Rh. ferrugineum* (Pisek 1950; Namibar & Fife 1991; Helmisaari 1995; Pornon et al. 1996; Lamaze et al. 2003; Marty et al. 2009; Marty et al. 2010). We therefore consider that a higher concentration of sugars, starch, nitrogen and a higher photosynthetic activity may favour the presence or predominance of fungi in cyl, whereas in pyl the same is favoured by a decrease in nutrients and photosynthetic activity. In contrast, the latter may be capable of destroying complex polymer cell wall compounds. Leaching substances in plants mostly include compounds of low molecular weight, sugars and amino acids (Tukey 1970), leading to a significant loss of nutritives (Wallace 1930; Schoch 1955), which are available on the leaf surface and may influence the epifoliar fungal community.

Generally, young and old leaves differ in their surface structure (Mechaber et al. 1996). While younger leaves are mostly strongly hydrophobic, this property may be lost in older leaves (Fogg 1947). Therefore leaching substances may have accumulated onto the surface of old leaves or already have disappeared. Nevertheless, different leaf age has been recognized as a factor of low relevance for fungal community composition ($R = 0.068$ in epifoliar communities) in this study. For subalpine and alpine plant species, snow cover is a requirement for survival in harsh environments (Körner & Larcher 1988). Due to its low thermal conductivity (Aulitzky et al. 1982; Rango & Martinec 1994), snow cover prevents temperature extremes exceeding frost tolerance levels, and snow-covered plants may therefore be exposed to temperatures close to 0 °C (Cernusca 1976). Winter desiccation is therefore not observed to occur (Sakai & Larcher 1987). The factor 'snow cover' probably explains to some degree the minimal impact of the factor 'leaf age' on the overall composition of the fungal assemblages on *Rh. ferrugineum.*

Based on the results of this study, we conclude that the epifoliar fungal community on *Rh. ferrugineum* is influenced by leaf age and sampling site, whereas the endofoliar fungal community is influenced by the vegetation zone, geographic region and sampling site.

Conclusion: residual and invading taxa

In the endofoliar habitat, two ecological groups were assumed to occur: a) those exhibiting a 'systemic' growth, combined with a presumably mutualistic relationship to the host plant, named 'residual endofoliar taxa' in the present study; and b) such groups, originating from the exterior of the leaf, named here 'invading endofoliar taxa'. *Rh. ferrugineum* is characterized by rather conspicuous glands on the lower leaf surface (Kratzmann 1910). The green-coloured glands closely cover the lower leaf surface of cyl and become physiologically inoperable in pyl. For some fungal species, they probably function as 'gateways' to the endophytic habitat. Among these taxa, *C. rhododendri*-1 may simply behave as an occasional invading endofoliar taxa, much more frequently remaining on the leaf surface. As we observed significant differences between the composition of the endophytic fungal community in the three altitudinal vegetation zones, the possibility of occasional infections of the leaf interior by *C. rhododendri*-1 is considered rather likely (Table 4). In contrast to *C. rhododendri*-1, *Cladosporium*-1 is regarded to be only an occasionally epifoliar fungus, more frequently occurring in the inner leaf tissues of *Rh. ferrugineum.* In summary, three types of fungi could be recognized. A group of epifoliar fungi exhibited the ability to form pigments (at least, in two of three culture media), and can be classified as 'residual epifoliar taxa'. A second group of endofoliar taxa may have colonized the leaf interior from the outer surface and accordingly obligately and facultatively exhibits hyphal wall pigmentation. They can be classified as 'invading endofoliar taxa'. A third group of strictly unpigmented taxa probably belongs to a group of 'residual endofoliar' taxa.

Due to the observed significant positive correlation between the properties of the leaf and hyphal pigmentation of the colonizing fungi, the potential to develop hyphal pigments is likely to have an indicative value for assigning these fungal taxa to major life strategies.

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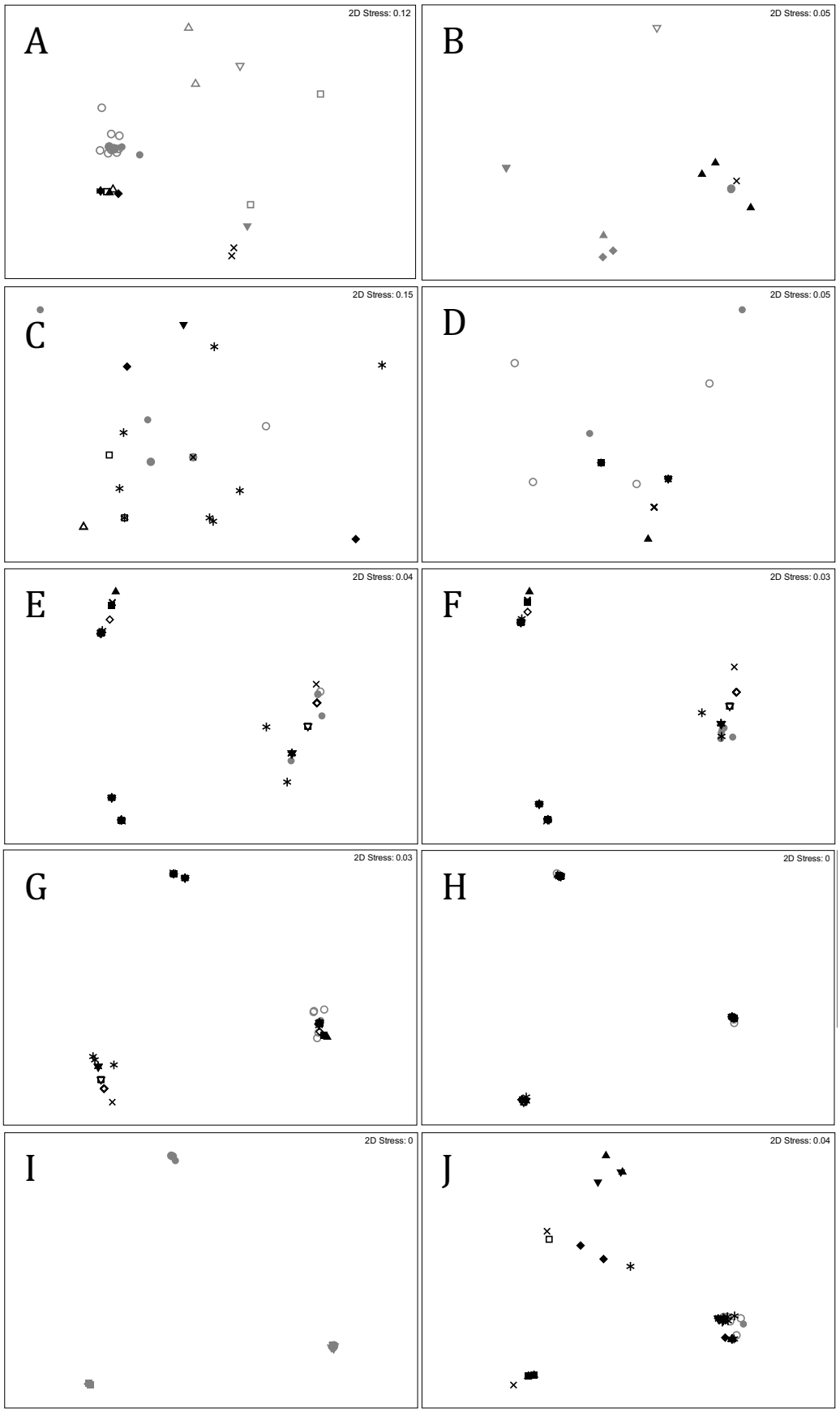
Figures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F
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G
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H
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I
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J
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Fig. 1 A. –O. Sequence similarities among groups of *Rhododendron ferrugineum*-associated fungal strains, and all published sequences showing at least 90% similarity to the respective group. Similarities among ITS rRNA gene sequences are visualized by non-metric multidimensional scaling (NMDS). Symbols code the origin of the corresponding strains: fungi associated with *Rh. ferrugineum*: [●] non-surface sterilized leaves of *Rh. ferrugineum*, [○] surface sterilized leaves of *Rh. ferrugineum*, [∗] surface sterilized living plant leaves or steams, [×] non-surface sterilized living plant material, [▼] leaf litter, [■] plant roots, [▲] soils, [△] rocks, [▽] air, [◇] water, [□] dust, [+] dung. Undifferentiated substrates [◆] include ants, lichens, wood, cheese, sea sediment and fungal fruit bodies. Other symbols in grey indicate related sequences with less than 90% similarity to the isolates. Letters indicate the different clusters according to the names of clusters used in the text. The groups include sequences deposited as members of the taxa *Sarcinomyces* (cluster A), Mycota (B), Dothideomycetes (C), *Herpotrichia juniperi* (D), *Cladosporium* (E–H), Vibrisseaceae (I), Leotiomycetes (J), *Capnocheirides rhododendri* (K), *Aureobasidium pullulans* (L), *Penicillium* (M), *Dothichiza pityophila* (N), *Hypoderma rubi* (O).

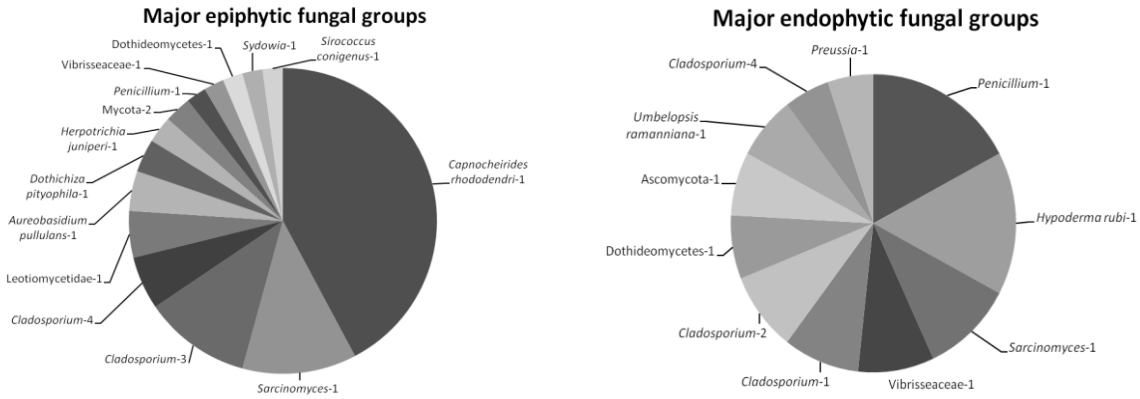

Fig. 2. Epiphytic and endophytic fungal OTUs (n = 3) on *Rhododendron ferrugineum.*

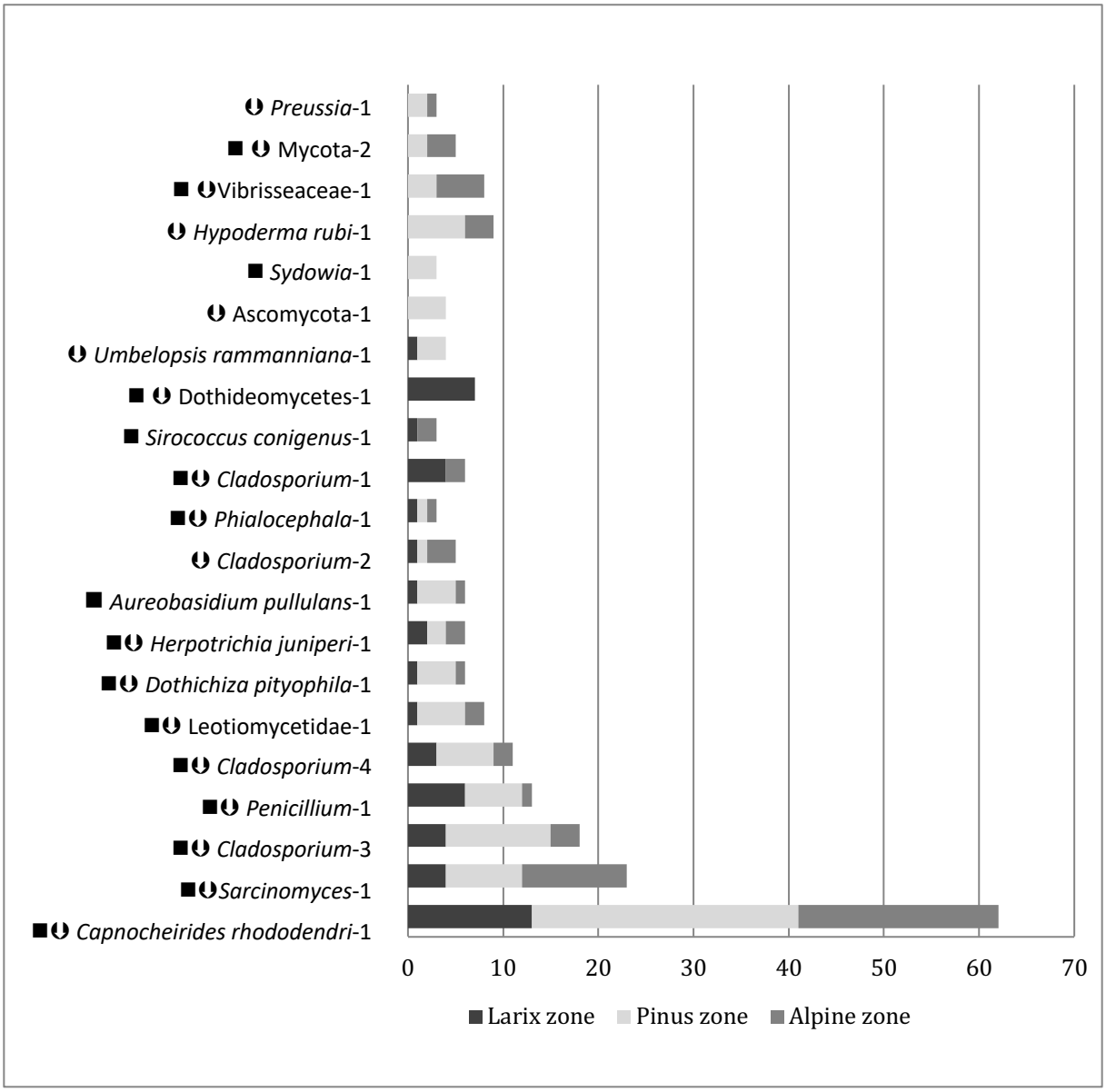

Fig. 3. Absolute abundance of the most abundant (n = 3) fungal OTUs associated with the leaves of *Rhododendron ferrugineum* in the different altitudinal vegetation zones. ■: OTUs exclusively occurring on the leaf surfaces (epiphytic fungi). 🕶: OTUs restricted to the leaf interior (endophytic fungi) are underlined. ■🕶: OTUs occurring as both epiphytic and endophytic.

Table 1. Substrates from which closely related sequences of the most frequent fungal OTUs (n = 5) were isolated, with reference to ecological data from GenBank.

OTU	Non-surface sterilized leaves of *Rh. ferrugineum*	Surface sterilized leaves of *Rh. ferrugineum*	Surface sterilized living plant leaves or steams	Non-surface sterilized living plant material	Leaf litter	Plant roots	Soils	Rocks	Air	Water	Dust	Dung	Undifferentiated substrates include ants, lichens, wood, cheese, sea sediment and fungal fruit bodies	Total
Aureobasidium pullulans-1	6	0	6	8	4	1	1	0	0	0	1	0	5	32
Capnocheirides rhododendri-1	60	2	2	2	3	1	3	3	1	0	0	0	3	82
Cladosporium-1	1	5	9	7	4	5	5	0	4	19	4	2	1	66
Cladosporium-2	0	5	9	7	4	5	5	0	4	19	4	2	1	65
Cladosporium-3	16	2	9	7	4	5	5	0	4	19	4	2	1	79
Cladosporium-4	8	3	9	7	4	5	5	0	4	19	4	2	1	72
Dothichiza pityophila-1	5	1	2	3	2	0	1	0	0	0	1	0	1	17
Dothideomycetes-1	3	4	8	1	1	0	0	2	0	0	2	0	2	23
Herpotrichia juniperi-1	4	2	0	19	2	1	1	0	0	0	0	0	2	31
Hypoderma rubi-1	0	9	1	5	0	0	3	0	0	0	0	0	10	28
Leotiomycetes-1	7	1	22	4	8	4	11	0	0	0	1	0	6	64
Mycota-2	4	1	0	1	3	0	4	0	1	0	0	0	2	16
Penicillium-1	3	10	3	3	2	0	1	0	3	1	1	0	13	40
Sarcinomyces-1	17	6	1	2	1	0	1	3	1	0	3	0	2	37
Vibrisseaceae-1	3	5	1	2	0	11	0	0	4	0	0	0	2	28

Table 2. Relative abundance of fungal OTUs (epifoliar and endofoliar fungi) isolated from *Rhododendron ferrugineum* in all (total) leaves, current year's leaves (cyl) and previous year's leaves (pyl).

OTU	**Epifoliar OTUs**			**Endofoliar OTUs**		
	total %	**cyl %**	**pyl %**	**total %**	**cyl %**	**pyl %**
Amphisphaeriaceae-1	2.2	0.0	3.8	0.0	0.0	0.0
Ascomycota-1	0.0	0.0	0.0	8.9	20.0	5.7
Ascomycota-3	2.2	2.4	1.9	0.0	0.0	0.0
Aureobasidium pullulans-1	6.5	0.0	11.5	0.0	0.0	0.0
Botryotinia-1	1.1	0.0	1.9	2.2	0.0	2.9
Capnocheirides rhododendri-1	64.5	82.9	50.0	4.4	0.0	5.7
Capnodiales-1	1.1	0.0	1.9	2.2	0.0	2.9
Cladosporium-1	1.1	0.0	1.9	11.1	0.0	14.3
Cladosporium-2	0.0	0.0	0.0	11.1	10.0	11.4
Cladosporium-3	17.2	2.4	28.8	4.4	10.0	2.9
Cladosporium-4	8.6	9.8	7.7	6.7	10.0	5.7
Coleophoma empetri-1	0.0	0.0	0.0	4.4	0.0	5.7
Dothichiza pityophila-1	5.4	4.9	5.8	2.2	0.0	2.9
Dothideomycetes-1	3.2	2.4	3.8	8.9	0.0	11.4
Dothioraceae-1	2.2	0.0	3.8	0.0	0.0	0.0
Geomyces-1	0.0	0.0	0.0	4.4	0.0	5.7
Herpotrichia juniperi-1	4.3	0.0	7.7	4.4	0.0	5.7
Hyaloscyphaceae-1	1.1	0.0	1.9	2.2	10.0	0.0
Hypocrea-1	2.2	0.0	3.8	0.0	0.0	0.0
Hypoderma rubi-1	0.0	0.0	0.0	20.0	0.0	25.7
Leotiomycetidae-1	7.5	2.4	11.5	2.2	0.0	2.9
Leotiomycetidae-2	2.2	0.0	3.8	0.0	0.0	0.0
Lewia infectoria-1	1.1	0.0	1.9	2.2	10.0	0.0
Mycota-2	4.3	0.0	7.7	2.2	0.0	2.9
Mycota-3	0.0	0.0	0.0	4.4	0.0	5.7
Mycota-4	1.1	0.0	1.9	2.2	0.0	2.9
Penicillium-1	3.2	2.4	3.8	22.2	40.0	17.1
Phaeosphaeria-2	2.2	0.0	3.8	0.0	0.0	0.0
Phialocephala-1	1.1	0.0	1.9	4.4	0.0	5.7
Phoma-1	2.2	0.0	3.8	0.0	0.0	0.0
Physalospora-1	0.0	0.0	0.0	4.4	0.0	5.7
Physalospora-2	0.0	0.0	0.0	4.4	0.0	5.7
Pleosporales-1	2.2	0.0	3.8	0.0	0.0	0.0
Pleosporales-3	2.2	0.0	3.8	0.0	0.0	0.0
Preussia-1	0.0	0.0	0.0	6.7	10.0	5.7
Preussia-2	0.0	0.0	0.0	4.4	0.0	5.7
Sarcinomyces-1	18.3	17.1	19.2	13.3	10.0	14.3
Sirococcus conigenus-1	3.2	0.0	5.8	0.0	0.0	0.0
Sydowia-1	3.2	0.0	5.8	0.0	0.0	0.0
Umbelopsis ramanniana-1	0.0	0.0	0.0	8.9	10.0	8.6
Vibrisseaceae-1	3.2	0.0	5.8	11.1	0.0	14.3

Table 3. Pigmentation of fungal OTUs isolated from *Rhododendron ferrugineum*.

Symbols indicate whether pigmentation is absent (○) or present (●) on malt yeast agar (MYA), carrot agar (C) and Rfer agar (agar with *Rh. ferrugineum* leaves). Strains were isolated from the leaf surface (■, epifoliar) or from the leaf interior (🌓, endofoliar).

Names Cluster	Ep/En	MYA	C	Rfer
Ascomycota-3	■	○	●	●
***Aureobasidium pullulans*-1**	■	○	●	●
Leotiomycetidae-2	■	○	●	●
***Phoma*-1**	■	●	●	●
***Sirococcus conigenus*-1**	■	○	○	●
***Sydowia*-1**	■	●	●	●
Ascomycota-1	🌓	●	●	●
***Cladosporium*-2**	🌓	●	●	●
***Coleophoma empetri*-1**	🌓	○	●	●
***Physalospora*-1**	🌓	○	○	○
***Physalospora*-2**	🌓	○	○	●
***Preussia*-1**	🌓	○	○	○
***Umbelopsis ramanniana*-1**	🌓	●	●	●
***Capnocheirides rhododendri*-1**	■ 🌓	●	●	●
***Cladosporium*-1**	■ 🌓	●	●	●
***Cladosporium*-3**	■ 🌓	○	●	●
***Cladosporium*-4**	■ 🌓	●	●	●
***Dothichiza pityophila*-1**	■ 🌓	●	●	●
Dothideomycetes-1	■ 🌓	●	●	●
***Herpotrichia juniperi*-1**	■ 🌓	●	●	●
Leotiomycetidae-1	■ 🌓	●	●	●
Mycota-2	■ 🌓	●	●	●
***Penicillium*-1**	■ 🌓	○	○	○
Sarcinomyces-1	■ 🌓	●	●	●
Vibrisseaceae-1	■ 🌓	○	●	●

Table 4. Effects of *Rhododendron ferrugineum* leaf age, alpine vegetation zone, sampling site and geographic location, as revealed by ANOSIM.

Factors	**Epifoliar OTUs**	**Endofoliar OTUs**
Leaf age	$p = 0.008$, $R = 0.068$	$p = 0.137$, $R = 0.045$
Alpine vegetation zone	$p = 0.578$, $R = –0.009$	$p = 0.016$, $R = 0.063$
Geographical region	$p = 0.362$, $R = 0.005$	$p = 0.001$, $R = 0.131$
Sampling site	$p = 0.009$, $R = 0.084$	$p = 0.001$, $R = 0.248$

Appendix

Appendix A: Details of deposition, assignment and grouping of the isolated fungal strains and the corresponding ITS nrDNA sequences. The first columns list the GenBank accession numbers for the ITS sequences and the ID of the corresponding strains deposited at the "Pilz-Referenz-Zentrum Jena" (FSU). Details of the most similar sequences found in GenBank are given in the following column. The assigned name is listed in the following column, together with details of the least well matched sequence for the name assignment. The total number of sequences considered (i.e. sequences obtaining "Bit Scores" which are at least 0.9 times as high as the "Bit Score" for the best matching sequence obtained), the number of environmental samples among them, and the number of outliers (i.e. sequences deposited under names not considered for the name assignment) are also given. The final column lists the name of the cluster in which the sequence is grouped in.

		Best matching sequence			**Name assignment**						
Sequence ID	**Culture ID**	**GenBank Acc.**	**Bit-Score**	**Deposited as**	**Number of sequences considered**	**Matches**	**Outliers**	**Environmental-samples**	**Lowest Bit-Score**	**Highest e-value**	**Cluster name (assigned consensus name)**
HQ228246		FJ791155.1	990	*Thanatephorus cucumeris*	50	26	0	24	963	0	Agaricomycetes-1
FR773218	FSU8682	EF619630.1	715	Amphisphaeriaceae sp.	22	19	1	2	680	0	Amphisphaeriaceae-1
FR773220	FSU10193	EF619630.1	688	Amphisphaeriaceae sp.	16	15	0	1	654	0	Amphisphaeriaceae-1
FR773329	FSU10381	GU062284.1	905	*Annulohypoxylon multiforme*	10	6	0	4	863	0	*Annulohypoxylon multiforme*-1
FR773221	FSU8681	FJ820752.1	874	Fungi sp.	4	3	0	1	789	0	*Ascochyta*-1
FR773212	FSU10412	AM084763.1	907	Ascomycete sp.	10	10	0	0	878	0	Ascomycete-2
HQ228238		AM999660.1	693	Fungi sp.	2	1	0	1	684	0	Ascomycota-1
HQ228244		AM999660.1	678	Fungi sp.	2	1	0	1	656	0	Ascomycota-1
HQ228322	FSU10440	AM999660.1	758	Fungi sp.	2	1	0	1	737	0	Ascomycota-1
HQ228343	FSU10394	AM999660.1	750	Fungi sp.	2	1	0	1	736	0	Ascomycota-1
FR773245		FJ553299.1	865	Ascomycota sp.	1	1	0	0	865	0	Ascomycota-3
FR773250		FJ903364.1	904	Ascomycota sp.	1	1	0	0	904	0	Ascomycota-3

FR773197		GQ376094.1	896	*Aureobasidium pullulans*	50	28	4	18	885	0	*Aureobasidium pullulans*-1
FR773204		EF690466.1	837	*Aureobasidium pullulans*	50	30	4	16	830	0	*Aureobasidium pullulans*-1
FR871187	FSU10198	EF690466.1	815	*Aureobasidium pullulans*	50	33	3	14	813	0	*Aureobasidium pullulans*-1
FR773317	FSU6491	GQ376094.1	920	*Aureobasidium pullulans*	50	25	4	21	905	0	*Aureobasidium pullulans*-1
FR773331		GQ376094.1	920	*Aureobasidium pullulans*	50	25	4	21	905	0	*Aureobasidium pullulans*-1
FR773411		EF690466.1	911	*Aureobasidium pullulans*	50	25	4	21	900	0	*Aureobasidium pullulans*-1
FR773400	FSU10611	FJ169673.1	837	*Botrytis* sp.	50	44	2	4	837	0	*Botryotinia*-1
HQ228335	FSU10393	FJ169667.2	850	*Botryotinia fuckeliana*	50	39	8	3	839	0	*Botryotinia*-1
FR773176	FSU8620	FJ553155.1	713	Mycosphaerellaceae	1	1	0	0	713	0	*Capnocheirides rhododendri*-1
FR773264		FJ553155.1	763	Mycosphaerellaceae	1	1	0	0	763	0	*Capnocheirides rhododendri*-1
FR773271		FJ553155.1	771	Mycosphaerellaceae	1	1	0	0	771	0	*Capnocheirides rhododendri*-1
FR773273		FJ553155.1	778	Mycosphaerellaceae	1	1	0	0	778	0	*Capnocheirides rhododendri*-1
FR773274		FJ553155.1	769	Mycosphaerellaceae	1	1	0	0	769	0	*Capnocheirides rhododendri*-1
FR773275		FJ553155.1	765	Mycosphaerellaceae	1	1	0	0	765	0	*Capnocheirides rhododendri*-1
FR871178		FJ553155.1	760	Mycosphaerellaceae	1	1	0	0	760	0	*Capnocheirides rhododendri*-1
FR773281	FSU10264	FJ553155.1	778	Mycosphaerellaceae	1	1	0	0	778	0	*Capnocheirides rhododendri*-1
FR773282		FJ553155.1	765	Mycosphaerellaceae	1	1	0	0	765	0	*Capnocheirides rhododendri*-1
FR773287		FJ553155.1	760	Mycosphaerellaceae	1	1	0	0	760	0	*Capnocheirides rhododendri*-1
FR773288		FJ553155.1	793	Mycosphaerellaceae	1	1	0	0	793	0	*Capnocheirides rhododendri*-1
FR773296		FJ553155.1	739	Mycosphaerellaceae	1	1	0	0	739	0	*Capnocheirides rhododendri*-1
FR773297		FJ553155.1	758	Mycosphaerellaceae	1	1	0	0	758	0	*Capnocheirides rhododendri*-1
FR773300	FSU8610	FJ553155.1	784	Mycosphaerellaceae	1	1	0	0	784	0	*Capnocheirides rhododendri*-1
FR871180		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR871181		FJ553155.1	767	Mycosphaerellaceae	1	1	0	0	767	0	*Capnocheirides rhododendri*-1
FR871182		FJ553155.1	763	Mycosphaerellaceae	1	1	0	0	763	0	*Capnocheirides rhododendri*-1
FR871183	FSU10377	FJ553155.1	754	Mycosphaerellaceae	1	1	0	0	754	0	*Capnocheirides rhododendri*-1
FR871188	FSU10265	FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR871191	FSU10378	FJ553155.1	761	Mycosphaerellaceae	1	1	0	0	761	0	*Capnocheirides rhododendri*-1
FR773304		FJ553155.1	776	Mycosphaerellaceae	1	1	0	0	776	0	*Capnocheirides rhododendri*-1
HQ228273	FSU10289	FJ553155.1	767	Mycosphaerellaceae	1	1	0	0	767	0	*Capnocheirides rhododendri*-1
FR773308		FJ553155.1	747	Mycosphaerellaceae	1	1	0	0	747	0	*Capnocheirides rhododendri*-1
FR773309		FJ553155.1	726	Mycosphaerellaceae	1	1	0	0	726	0	*Capnocheirides rhododendri*-1
FR773310	FSU8607	FJ553155.1	787	Mycosphaerellaceae	1	1	0	0	787	0	*Capnocheirides rhododendri*-1
FR773311	FSU8608	FJ553155.1	774	Mycosphaerellaceae	1	1	0	0	774	0	*Capnocheirides rhododendri*-1
FR773324	FSU8571	FJ553155.1	741	Mycosphaerellaceae	1	1	0	0	741	0	*Capnocheirides rhododendri*-1
FR773325		FJ553155.1	750	Mycosphaerellaceae	1	1	0	0	750	0	*Capnocheirides rhododendri*-1
FR773326	FSU10267	FJ553155.1	728	Mycosphaerellaceae	1	1	0	0	728	0	*Capnocheirides rhododendri*-1
FR773332		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773333		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773334		FJ553155.1	785	Mycosphaerellaceae	1	1	0	0	785	0	*Capnocheirides rhododendri*-1
FR773335		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773337		FJ553155.1	785	Mycosphaerellaceae	1	1	0	0	785	0	*Capnocheirides rhododendri*-1
FR773361		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773362		FJ553155.1	791	Mycosphaerellaceae	1	1	0	0	791	0	*Capnocheirides rhododendri*-1

FR773363		FJ553155.1	715	Mycosphaerellaceae	1	1	0	0	715	0	*Capnocheirides rhododendri*-1
FR773364		FJ553155.1	760	Mycosphaerellaceae	1	1	0	0	760	0	*Capnocheirides rhododendri*-1
FR773379	FSU8574	FJ553155.1	745	Mycosphaerellaceae	1	1	0	0	745	0	*Capnocheirides rhododendri*-1
FR773380		FJ553155.1	761	Mycosphaerellaceae	1	1	0	0	761	0	*Capnocheirides rhododendri*-1
FR773381	FSU10269	FJ553155.1	752	Mycosphaerellaceae	1	1	0	0	752	0	*Capnocheirides rhododendri*-1
FR773383		FJ553155.1	791	Mycosphaerellaceae	1	1	0	0	791	0	*Capnocheirides rhododendri*-1
FR773389	FSU8575	FJ553155.1	773	Mycosphaerellaceae	1	1	0	0	773	0	*Capnocheirides rhododendri*-1
FR773390		FJ553155.1	806	Mycosphaerellaceae	1	1	0	0	806	0	*Capnocheirides rhododendri*-1
FR773393	FSU8603	FJ553155.1	704	Mycosphaerellaceae	1	1	0	0	704	0	*Capnocheirides rhododendri*-1
FR773394		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773395		FJ553155.1	793	Mycosphaerellaceae	1	1	0	0	793	0	*Capnocheirides rhododendri*-1
FR773396		FJ553155.1	765	Mycosphaerellaceae	1	1	0	0	765	0	*Capnocheirides rhododendri*-1
FR773405		FJ553155.1	758	Mycosphaerellaceae	1	1	0	0	758	0	*Capnocheirides rhododendri*-1
FR773442		FJ553155.1	784	Mycosphaerellaceae	1	1	0	0	784	0	*Capnocheirides rhododendri*-1
FR773443		FJ553155.1	795	Mycosphaerellaceae	1	1	0	0	795	0	*Capnocheirides rhododendri*-1
FR773444	FSU10274	FJ553155.1	773	Mycosphaerellaceae	1	1	0	0	773	0	*Capnocheirides rhododendri*-1
FR773461		FJ553155.1	704	Mycosphaerellaceae	1	1	0	0	704	0	*Capnocheirides rhododendri*-1
FR773462		FJ553155.1	773	Mycosphaerellaceae	1	1	0	0	773	0	*Capnocheirides rhododendri*-1
FR773464		FJ553155.1	765	Mycosphaerellaceae	1	1	0	0	765	0	*Capnocheirides rhododendri*-1
FR773466		FJ553155.1	769	Mycosphaerellaceae	1	1	0	0	769	0	*Capnocheirides rhododendri*-1
FR773467		FJ553155.1	758	Mycosphaerellaceae	1	1	0	0	758	0	*Capnocheirides rhododendri*-1
FR773468		FJ553155.1	721	Mycosphaerellaceae	1	1	0	0	721	0	*Capnocheirides rhododendri*-1
FR773470		FJ553155.1	739	Mycosphaerellaceae	1	1	0	0	739	0	*Capnocheirides rhododendri*-1
FR773473		FJ553155.1	778	Mycosphaerellaceae	1	1	0	0	778	0	*Capnocheirides rhododendri*-1
FR773481		FJ553155.1	708	Mycosphaerellaceae	1	1	0	0	708	0	*Capnocheirides rhododendri*-1
HQ228306		FJ553155.1	760	Mycosphaerellaceae	1	1	0	0	760	0	*Capnocheirides rhododendri*-1
FR773295	FSU8566	EU167586.1	579	*Cladosporium* sp.	9	7	0	2	551	6.00E-162	*Capnodialea*-2
HQ228268	FSU10374	AM992158.2	752	Capnodiales sp.	4	3	0	1	737	0	Capnodiales-1
FR773213		AM992160.1	813	Capnodiales sp.	4	3	0	1	778	0	Capnodiales-1
FR773398		AM992160.1	686	Capnodiales sp.	4	3	0	1	656	0	Capnodiales-3
HQ228270		GQ996574.1	937	*Chaetomium funicola*	15	9	0	6	894	0	*Chaetomium*-1
FR773313	FSU10420	FJ820813.1	881	Fungi sp.	5	2	1	2	843	0	*Chalara microchona*-1
HQ228251		FJ266012.1	813	Fungi sp.	50	50	0	0	813	0	*Cladosporium*-1
HQ228291	FSU10211	FJ266011.1	791	Fungi sp.	50	50	0	0	791	0	*Cladosporium*-1
FR773477		FJ266011.1	809	Fungi sp.	50	50	0	0	809	0	*Cladosporium*-1
HQ228323	FSU10282	FJ266011.1	869	Fungi sp.	50	50	0	0	869	0	*Cladosporium*-1
FR773492		EU977278.1	869	Fungi sp.	3	3	0	0	869	0	*Cladosporium*-1
HQ228334	FSU10391	FJ266011.1	857	Fungi sp.	50	50	0	0	857	0	*Cladosporium*-1
HQ228254		EU977278.1	789	Fungi sp.	3	3	0	0	789	0	*Cladosporium*-2
FR773174		EU977278.1	791	Fungi sp.	3	3	0	0	791	0	*Cladosporium*-2
HQ228260		EU977278.1	791	Fungi sp.	3	3	0	0	791	0	*Cladosporium*-2
HQ228261		EU977278.1	876	Fungi sp.	3	3	0	0	876	0	*Cladosporium*-2
HQ228321	FSU10204	EU977278.1	869	Fungi sp.	3	3	0	0	869	0	*Cladosporium*-2
FR773178		EU715666.1	678	*Cladosporium* sp.	50	42	3	0	678	0	*Cladosporium*-3

FR773179		EU715666.1	837	*Cladosporium* sp.	50	43	2	5	837	0	*Cladosporium*-3
FR773194		EU715666.1	881	*Cladosporium* sp.	50	43	2	5	881	0	*Cladosporium*-3
FR773217		EU715666.1	865	*Cladosporium* sp.	50	43	2	5	865	0	*Cladosporium*-3
FR773228		EU715666.1	854	*Cladosporium* sp.	50	43	2	5	854	0	*Cladosporium*-3
FR773236		EU715666.1	880	*Cladosporium* sp.	50	44	2	4	880	0	*Cladosporium*-3
FR773247		EU715666.1	885	*Cladosporium* sp.	50	44	2	4	885	0	*Cladosporium*-3
FR773265	FSU10209	EU715666.1	802	*Cladosporium* sp.	50	43	2	5	802	0	*Cladosporium*-3
FR773291		EU715666.1	752	*Cladosporium* sp.	50	39	2	9	752	0	*Cladosporium*-3
FR773344		EU715666.1	850	*Cladosporium* sp.	50	43	2	5	850	0	*Cladosporium*-3
FR773387		EU715666.1	874	*Cladosporium* sp.	50	43	2	5	874	0	*Cladosporium*-3
FR773419		EU715666.1	845	*Cladosporium* sp.	50	43	2	5	845	0	*Cladosporium*-3
FR773420		EU715666.1	833	*Cladosporium* sp.	50	43	2	5	833	0	*Cladosporium*-3
FR773425	FSU8555	EU715666.1	881	*Cladosporium* sp.	50	43	2	5	881	0	*Cladosporium*-3
FR773428	FSU10271	EU715666.1	867	*Cladosporium* sp.	50	43	2	5	867	0	*Cladosporium*-3
FR773483		AY251077.2	784	*Cladosporium sphaer-ospermum*	50	43	2	5	780	0	*Cladosporium*-3
HQ228312	FSU10214	EU715666.1	874	*Cladosporium* sp.	50	43	2	5	874	0	*Cladosporium*-3
HQ228332		EF504369.1	863	Fungi sp.	50	42	2	6	861	0	*Cladosporium*-3
FR773195	FSU8559	FJ556911.1	802	*Cladosporium cladospori-oides*	50	29	5	16	802	0	*Cladosporium*-4
FR773199		GQ370370.1	846	*Cladosporium* sp.	50	29	5	16	846	0	*Cladosporium*-4
FR773216		GQ370370.1	852	*Cladosporium* sp.	50	29	6	15	852	0	*Cladosporium*-4
FR773227		EU715666.1	837	*Cladosporium* sp.	50	43	2	5	837	0	*Cladosporium*-4
FR773244		AY251071.2	878	*Cladosporium uredinicola*	50	26	7	17	878	0	*Cladosporium*-4
FR773343	FSU8549	GU212392.1	872	*Cladosporium* sp.	50	21	8	21	872	0	*Cladosporium*-4
FR773386	FSU10270	AY251071.2	850	*Cladosporium uredinicola*	50	28	5	17	845	0	*Cladosporium*-4
FR773410	FSU8554	EF672315.1	887	*Cladosporium* sp.	50	22	8	20	881	0	*Cladosporium*-4
HQ228285	FSU8557	GU214631.1	887	*Cladosporium* sp.	50	24	7	19	887	0	*Cladosporium*-4
HQ228308	FSU10213	GU214631.1	894	*Cladosporium* sp.	50	24	7	19	894	0	*Cladosporium*-4
HQ228328		GU214631.1	852	*Cladosporium* sp.	50	24	7	19	852	0	*Cladosporium*-4
HQ228266	FSU8616	EU686928.1	614	Fungi sp.	4	1	0	3	612	2.00E-172	*Clypeosphaeria mamillana*-1
HQ228265	FSU8617	DQ979647.1	795	Fungi sp.	37	33	2	2	758	0	*Coleophoma empetri*-1
HQ228278	FSU8553	FJ480139.1	750	*Coleophoma empetri*	41	36	2	3	723	0	*Coleophoma empetri*-1
FR773341		AJ244242.1	902	*Dothichiza pityophila*	2	1	1	0	885	0	*Dothichiza pityophila*-1
FR773423		FJ553079.1	1007	Dothioraceae sp.	2	1	1	0	959	0	*Dothichiza pityophila*-1
FR773427		AJ244242.1	963	*Dothichiza pityophila*	2	1	1	0	961	0	*Dothichiza pityophila*-1
FR773434	FSU10386	AJ244242.1	957	*Dothichiza pityophila*	2	1	1	0	946	0	*Dothichiza pityophila*-1
HQ228284	FSU8576	AJ244242.1	952	*Dothichiza pityophila*	2	1	1	0	946	0	*Dothichiza pityophila*-1
FR773482		AJ244242.1	911	*Dothichiza pityophila*	2	1	1	0	900	0	*Dothichiza pityophila*-1
HQ228239	FSU10191	AM901920.1	891	Fungi sp.	10	3	0	7	850	0	Dothideomycetes-1
HQ228241		AM901920.1	872	Fungi sp.	14	4	0	10	830	0	Dothideomycetes-1
HQ228271		FJ997287.1	926	Dothideales sp.	6	3	0	3	880	0	Dothideomycetes-1
FR773237		FJ997287.1	926	Dothideales sp.	6	3	0	3	880	0	Dothideomycetes-1
FR773399	FSU10383	AM901920.1	880	Fungi sp.	13	4	0	9	837	0	Dothideomycetes-1
FR773407	FSU10384	FJ997287.1	902	Dothideales sp.	8	4	0	4	857	0	Dothideomycetes-1
HQ228277		AM901920.1	941	Fungi sp.	7	2	0	5	894	0	Dothideomycetes-1

FR773202	FSU10409	FJ150873.1	595	*Kabatiella microsticta*	28	9	0	19	568	6.00E-167	Dothioraceae-1
FR773415		FJ612670.1	734	Fungi sp.	24	4	0	20	701	0	Dothioraceae-1
FR871179	FSU8609	DQ667153.1	835	*Exobasidium rhododendri*	2	2	0	0	822	0	*Exobasidium rhododendri*-1
HQ228236		EF540755.1	837	*Geomyces pannorum*	32	16	0	16	798	0	*Geomyces*-1
HQ228252		EF540755.1	769	*Geomyces pannorum*	32	16	0	16	736	0	*Geomyces*-1
HQ228295	FSU10348	AY465448.1	832	Helotiaceae sp.	2	2	0	0	822	0	Helotiaceae-1
FR773312	FSU10419	AY969380.1	732	Fungi sp.	10	2	2	6	697	0	Helotiales-1
HQ228240	FSU10295	FJ904465.1	798	*Herpotrichia juniperi*	45	34	1	10	760	0	*Herpotrichia juniperi*-1
FR773198	FSU10263	FJ904461.1	800	*Herpotrichia juniperi*	45	34	1	10	767	0	*Herpotrichia juniperi*-1
FR773205	FSU8561	GQ203759.1	730	*Herpotrichia juniperi*	50	38	1	11	702	0	*Herpotrichia juniperi*-1
FR773286	FSU10197	FJ904454.1	826	*Herpotrichia juniperi*	50	41	1	8	787	0	*Herpotrichia juniperi*-1
FR871184		FJ904484.1	806	*Herpotrichia juniperi*	50	41	1	8	784	0	*Herpotrichia juniperi*-1
HQ228327		GQ203759.1	761	*Herpotrichia juniperi*	50	40	1	9	739	0	*Herpotrichia juniperi*-1
FR773203	FSU8560	X74923.1	937	*Heterobasidion annosum*	50	50	0	0	928	0	*Heterobasidion*-1
FR773231	FSU8684	FJ379833.1	745	*Hyalodendriella* sp.	3	1	1	0	721	0	*Hyalodendriella*-1
FR773330	FSU8548	U59145.1	841	*Lachnellula calyciformis*	3	2	1	0	802	0	Hyaloscyphaceae-1
HQ228303	FSU10435	U59145.1	856	*Lachnellula calyciformis*	2	2	0	0	837	0	Hyaloscyphaceae-1
FR773201	FSU8619	AM504125.1	1051	*Hypholoma fasciculare*	10	10	0	0	1013	0	*Hypholoma fasciculare*-1
FR773185	FSU10338	EU294196.1	780	*Hypocrea* sp.	24	16	2	6	741	0	*Hypocrea*-1
FR773222	FSU8544	DQ083026.1	1000	*Trichoderma croceum*	40	28	8	4	950	0	*Hypocrea*-1
HQ228287	FSU10429	GU367895.1	628	*Hypoderma rubi*	32	15	11	6	597	6.00E-177	*Hypoderma rubi*-1
FR773456		GU367895.1	651	*Hypoderma rubi*	31	14	11	6	619	0	*Hypoderma rubi*-1
FR773484		GU367895.1	656	*Hypoderma rubi*	30	14	10	6	625	0	*Hypoderma rubi*-1
HQ228316		GU367895.1	612	*Hypoderma rubi*	33	15	12	6	584	6.00E-172	*Hypoderma rubi*-1
FR773493		GU367898.1	612	*Hypoderma rubi*	32	15	11	6	584	6.00E-172	*Hypoderma rubi*-1
HQ228336		GU367895.1	601	*Hypoderma rubi*	37	15	15	6	573	1.00E-168	*Hypoderma rubi*-1
HQ228337		GU367895.1	612	*Hypoderma rubi*	27	15	9	3	586	6.00E-172	*Hypoderma rubi*-1
HQ228340		GU367895.1	597	*Hypoderma rubi*	39	15	18	6	568	2.00E-167	*Hypoderma rubi*-1
HQ228344		GU367895.1	641	*Hypoderma rubi*	32	14	12	6	612	0	*Hypoderma rubi*-1
FR773232	FSU10375	FJ612670.1	734	Fungi sp.	3	1	0	2	704	0	*Kabatiella caulivora*-1
HQ228297	FSU10432	FJ528692.1	1271	Fungi sp.	21	17	0	4	1212	0	*Leohumicola*-1
FR773370	FSU10422	FJ553685.1	839	Leotiomycetes sp.	7	5	1	0	821	0	Leotiomycetes-3
FR773314	FSU8606	DQ273332.1	536	Pezizomycotina sp.	13	9	1	3	510	4.00E-149	Leotiomycetes-4
FR773182		EU625294.1	865	Fungi sp.	30	15	4	11	822	0	Leotiomycetidae-1
FR773184	FSU8558	AY183372.1	832	Cf. *Phoma* sp.	11	1	3	7	795	0	Leotiomycetidae-1
FR773188	FSU10261	FJ904499.1	826	*Allantophomopsis* sp.	28	15	4	9	787	0	Leotiomycetidae-1
FR773257	FSU10195	AY969742.1	856	Fungi sp.	30	15	5	10	821	0	Leotiomycetidae-1
FR773267		EU625294.1	856	Fungi sp.	29	14	5	10	817	0	Leotiomycetidae-1
FR773277		AY969742.1	880	Fungi sp.	29	15	5	9	839	0	Leotiomycetidae-1
FR773435	FSU10272	EU625294.1	833	Fungi sp.	30	15	6	9	793	0	Leotiomycetidae-1
HQ228310		FJ904499.1	773	*Allantophomopsis* sp.	35	16	6	13	739	0	Leotiomycetidae-1
FR773457	FSU10291	FJ904499.1	806	*Allantophomopsis* sp.	36	18	6	12	767	0	Leotiomycetidae-10
FR773193		FJ904499.1	789	*Allantophomopsis* sp.	33	15	5	13	761	0	Leotiomycetidae-2
FR773200	FSU10288	AY608648.1	846	*Phacidiopycnis washing-tonensis*	35	18	4	13	811	0	Leotiomycetidae-2

FR773206	FSU8562	FJ904499.1	747	*Allantophomopsis* sp.	36	16	6	14	713	0	Leotiomycetidae-3
FR773226		FJ904499.1	815	*Allantophomopsis* sp.	33	15	6	12	782	0	Leotiomycetidae-4
FR773251		AY969742.1	856	Fungi sp.	35	18	5	12	821	0	Leotiomycetidae-5
FR871186		AY969742.1	854	Fungi sp.	35	18	5	12	819	0	Leotiomycetidae-6
FR773436		FJ904499.1	800	*Allantophomopsis* sp.	33	15	6	12	767	0	Leotiomycetidae-8
FR773438		FJ904499.1	826	*Allantophomopsis* sp.	36	18	6	12	785	0	Leotiomycetidae-9
HQ228245	FSU10207	GQ376103.1	918	*Lewia infectoria*	50	33	13	4	907	0	*Lewia infectoria*-1
FR773302	FSU6492	GQ376103.1	846	*Lewia infectoria*	50	29	11	10	839	0	*Lewia infectoria*-1
FR773375	FSU8680	DQ491498.1	1029	*Mollisia cinerea*	1	1	0	0	1029	0	*Mollisia cinerea*-1
HQ228313	FSU10438	AF439461.1	776	*Leptosphaeria dryadis*	4	3	1	0	745	0	*Monodictys arctica*-1
FR773283	FSU10341	DQ068346.1	817	Fungi sp.	18	17	0	1	778	0	*Mycosphaerella*-1
HQ228253	FSU10407	EF619925.1	856	*Mycosphaerella* sp.	4	2	1	0	845	0	*Mycosphaerella*-2
FR773319		EF434011.1	856	Fungi sp.	1	1	0	0	856	0	Mycota-1
FR773397	FSU8602	FJ612953.1	303	Fungi sp.	4	4	0	0	303	4.00E-79	Mycota-10
HQ228279	FSU10426	AM901933.1	869	Fungi sp.	11	11	0	0	832	0	Mycota-11
HQ228280	FSU10427	AM999755.1	1315	Fungi sp.	2	2	0	0	1308	0	Mycota-12
HQ228281	FSU10346	EF619862.1	444	Fungi sp.	1	1	0	0	444	2.00E-121	Mycota-13
FR773451	FSU10428	AM999599.1	702	Fungi sp.	1	1	0	0	702	0	Mycota-14
HQ228255	FSU10586	FJ820750.1	920	Fungi sp.	1	1	0	0	920	0	Mycota-15
FR871193	FSU10347	EU516950.1	734	Fungi sp.	2	2	0	0	710	0	Mycota-19
FR773269		AM999660.1	773	Fungi sp.	2	2	0	0	741	0	Mycota-2
FR773316	FSU8569	AM999660.1	743	Fungi sp.	2	2	0	0	773	0	Mycota-2
FR773371		AM999660.1	765	Fungi sp.	2	2	0	0	728	0	Mycota-2
FR773421	FSU10385	AM999660.1	774	Fungi sp.	2	2	0	0	774	0	Mycota-2
HQ228298		AM999660.1	732	Fungi sp.	2	2	0	0	710	0	Mycota-2
HQ228307	FSU10436	FJ235861.1	809	Fungi sp.	4	4	0	0	778	0	Mycota-20
HQ228339	FSU10205	AY561199.1	641	Fungi sp.	1	1	0	0	641	1.00E-180	Mycota-3
HQ228345	FSU10613	AY561199.1	612	Fungi sp.	1	1	0	0	612	8.00E-172	Mycota-3
FR773219	FSU10414	FJ820775.1	778	Fungi sp.	2	2	0	0	778	0	Mycota-4
HQ228346	FSU10423	EU686068.1	715	Fungi sp.	2	2	0	0	713	0	Mycota-4
FR773223	FSU10415	EU517039.1	848	Fungi sp.	2	2	0	0	846	0	Mycota-6
HQ228272	FSU10417	FJ820826.1	963	Fungi sp.	1	1	0	0	963	0	Mycota-7
FR773292	FSU10608	AM262385.1	691	Fungi sp.	1	1	0	0	691	0	Mycota-8
FR773360	FSU10609	DQ884464.1	512	Fungi sp.	1	1	0	0	512	6.00E-142	Mycota-9
FR773214	FSU10413	AY969346.1	507	Fungi sp.	50	17	16	17	483	3.00E-140	*Neofabraea alba*-1
HQ228309	FSU10437	AF201751.1	1064	*Nodulisporium* sp.	3	3	0	0	1062	0	*Nodulisporium*-1
HQ228311		EU781661.1	466	*Nodulisporium* sp.	2	2	0	0	466	7.00E-128	*Nodulisporium*-2
FR773229		EU128597.1	900	*Penicillium glabrum*	50	30	14	6	881	0	*Penicillium glabrum*-1
FR773211		EU729705.1	880	*Penicillium citreonigrum*	46	43	0	3	837	0	*Penicillium*-1
FR773225		EU729705.1	902	*Penicillium citreonigrum*	45	42	0	3	857	0	*Penicillium*-1
FR773385		EU729705.1	900	*Penicillium citreonigrum*	43	40	0	3	869	0	*Penicillium*-1
HQ228290	FSU10275	EU729705.1	854	*Penicillium citreonigrum*	47	43	1	3	813	0	*Penicillium*-1
HQ228317		EU128641.1	850	*Penicillium citreonigrum*	50	46	1	3	808	0	*Penicillium*-1
HQ228318	FSU10281	EU128641.1	929	*Penicillium citreonigrum*	50	46	1	3	887	0	*Penicillium*-1

HQ228319		EU128641.1	848	*Penicillium citreonigrum*	50	46	1	3	806	0	*Penicillium*-1
HQ228320		EU128641.1	920	*Penicillium citreonigrum*	50	46	1	3	878	0	*Penicillium*-1
HQ228325	FSU10283	EU128641.1	904	*Penicillium citreonigrum*	50	46	1	3	861	0	*Penicillium*-1
HQ228329		EU128641.1	887	*Penicillium citreonigrum*	50	46	1	3	845	0	*Penicillium*-1
FR773497		EU128641.1	826	*Penicillium citreonigrum*	46	43	0	3	789	0	*Penicillium*-1
HQ228333	FSU10286	EU128641.1	898	*Penicillium citreonigrum*	50	46	1	3	856	0	*Penicillium*-1
HQ228338		EU128641.1	878	*Penicillium citreonigrum*	50	46	1	3	835	0	*Penicillium*-1
FR773315	FSU8547	FJ820605.1	994	Fungi sp.	7	3	0	4	963	0	*Peniophora incarnata*-1
FR773368	FSU10610	GU433224.1	963	*Peniophora* sp.	20	8	1	11	915	0	*Peniophora*-1
FR773422	FSU10612	AM901741.1	920	Fungi sp.	2	1	0	1	918	0	*Phaeococcomyces nigricans*-1
FR773278	FSU10418	FJ609291.1	833	Fungi sp.	40	20	3	17	793	0	*Phaeosphaeria*-1
FR773369	FSU8552	AF439478.1	828	*Phaeosphaeria dennisiana*	5	5	0	0	797	0	*Phaeosphaeria*-2
FR773418		AF439496.1	865	*Phaeosphaeria padellana*	4	4	0	0	826	0	*Phaeosphaeria*-2
HQ228242		FJ903314.1	652	*Phialocephala* sp.	18	14	0	4	623	0	*Phialocephala*-1
FR773376	FSU10345	DQ309109.1	706	Fungi sp.	18	13	0	5	673	0	*Phialocephala*-1
FR773488	FSU10216	FJ903314.1	658	*Phialocephala* sp.	19	14	0	5	632	0	*Phialocephala*-1
FR773189	FSU10339	AB465199.1	774	*Phoma* sp.	18	16	0	2	736	0	*Phoma complanata*-1
FR773328	FSU10344	FJ515608.1	553	*Phoma complanata*	1	1	0	0	553	4.00E-154	*Phoma complanata*-1
FR773412		EF589893.1	856	*Phoma* sp.	1	1	0	0	856	0	*Phoma*-1
FR773414	FSU6494	EF589893.1	833	*Phoma* sp.	1	1	0	0	833	0	*Phoma*-1
FR773192	FSU10605	AJ279473.1	830	Ascomycete sp.	8	3	0	5	795	0	*Phoma*-2
FR773165	FSU10192	FJ603599.1	374	*Physalospora vaccinii*	21	20	1	0	357	2.00E-97	*Physalospora*-1
HQ228283		FJ603599.1	372	*Physalospora vaccinii*	21	20	1	0	357	3.00E-100	*Physalospora*-1
HQ228248		FJ603599.1	388	*Physalospora vaccinii*	6	6	0	0	372	1.00E-99	*Physalospora*-2
HQ228275	FSU8573	FJ603610.1	364	*Physalospora vaccinii*	20	20	0	0	350	1.00E-99	*Physalospora*-2
HQ228282		FJ603599.1	292	*Physalospora vaccinii*	20	19	1	0	278	8.00E-76	*Physalospora*-3
FR773180		EU852362.1	898	*Leptosphaeria* sp.	4	2	2	0	854	0	Pleosporales-1
FR773224	FSU8685	EU852362.1	939	*Leptosphaeria* sp.	2	2	0	0	922	0	Pleosporales-1
FR773215	FSU8678	U04207.1	771	*Leptosphaeria doliolum*	2	2	0	0	754	0	Pleosporales-2
FR773230	FSU8685	FJ427022.1	848	*Phoma herbarum*	29	14	4	11	806	0	Pleosporales-3
FR773252	FSU8563	FJ554029.1	874	Fungi sp.	50	34	10	6	833	0	Pleosporales-3
FR773186	FSU10604	FJ515608.1	776	*Phoma complanata*	50	28	5	17	739	0	Pleosporales-4
HQ228247		FJ210518.1	743	*Preussia* sp.	21	6	12	3	710	0	*Preussia*-1
HQ228267	FSU10208	AY510415.1	863	*Preussia intermedia*	8	6	2	0	828	0	*Preussia*-1
HQ228326		AY510415.1	848	*Preussia intermedia*	8	6	2	0	821	0	*Preussia*-1
HQ228269		GQ203775.1	725	*Preussia borealis*	9	6	3	0	697	0	*Preussia*-2
HQ228296	FSU10212	GQ203775.1	778	*Preussia borealis*	9	6	3	0	756	0	*Preussia*-2
HQ228262	FSU10408	DQ068995.1	870	*Pseudeurotium bakeri*	13	8	0	5	833	0	*Pseudeurotium*-1
HQ228292		AY128700.1	647	*Pseudotaeniolina globosa*	1	1	0	0	647	0	*Pseudotaeniolina globosa*-1
FR773246	FSU8614	GU328618.1	959	Basidiomycota sp.	2	1	0	1	941	0	*Psilocybe montana*-1
FR773301	FSU8568	EF151248.1	957	*Rhodotorula psychrophe-nolica*	3	3	0	0	941	0	*Rhodotorula psychrophenolica*-1
HQ228342	FSU10442	EF040837.1	394	Fungi sp.	16	7	3	6	375	3.00E-106	*Rhodotorula*-1
FR773303	FSU10343	AF384681.1	1042	*Rhynchosporium secalis*	6	6	0	0	1037	0	*Rhynchosporium secalis*-1
FR773290	FSU10342	AY744286.1	603	Leptosphaeriaceae sp.	12	5	1	6	573	4.00E-169	*Saccharicola*-1

FR773431		U04203.1	540	*Saccharicola bicolor*	15	6	1	8	514	3.00E-150	*Saccharicola*-2
HQ228237		AY843045.1	518	*Stigmina* sp.	9	2	1	6	494	1.00E-143	*Sarcinomyces*-1
HQ228258		AY843192.1	621	Fungi sp.	4	2	0	2	604	8.00E-175	*Sarcinomyces*-1
HQ228263		AY843192.1	606	Fungi sp.	4	2	0	0	623	0	*Sarcinomyces*-1
FR773272	FSU8613	FJ553309.1	621	Fungi sp.	4	2	0	2	608	8.00E-175	*Sarcinomyces*-1
FR773276		FJ553309.1	638	Fungi sp.	4	2	0	2	625	8.00E-180	*Sarcinomyces*-1
FR773298		AY843192.1	599	Fungi sp.	4	2	0	2	582	4.00E-168	*Sarcinomyces*-1
FR773299	FSU8611	AY843192.1	630	Fungi sp.	4	2	0	2	617	1.00E-177	*Sarcinomyces*-1
FR871189		AY843192.1	582	Fungi sp.	4	2	0	2	566	4.00E-163	*Sarcinomyces*-1
FR871190		AY843192.1	590	Fungi sp.	4	2	0	2	573	2.00E-165	*Sarcinomyces*-1
FR773307	FSU10379	AY843192.1	636	Fungi sp.	4	2	0	2	619	3.00E-179	*Sarcinomyces*-1
FR773322	FSU10380	AY843192.1	630	Fungi sp.	4	2	0	2	619	1.00E-177	*Sarcinomyces*-1
FR773339		AY843192.1	577	Fungi sp.	5	2	1	0	544	2.00E-161	*Sarcinomyces*-1
FR773377		FJ553309.1	651	Fungi sp.	4	2	0	2	636	0	*Sarcinomyces*-1
FR773378		AY843192.1	625	Fungi sp.	4	2	0	2	608	6.00E-176	*Sarcinomyces*-1
FR773391	FSU10382	AY843192.1	630	Fungi sp.	4	2	0	2	619	1.00E-177	*Sarcinomyces*-1
FR773392		AY843192.1	636	Fungi sp.	4	2	0	2	625	3.00E-179	*Sarcinomyces*-1
FR773406		AY843192.1	628	Fungi sp.	4	2	0	2	612	5.00E-177	*Sarcinomyces*-1
FR773408		AY843192.1	628	Fungi sp.	4	2	0	2	617	5.00E-177	*Sarcinomyces*-1
FR773441		FJ553309.1	651	Fungi sp.	4	2	0	2	630	0	*Sarcinomyces*-1
FR773472		AY843192.1	630	Fungi sp.	4	2	0	2	619	1.00E-177	*Sarcinomyces*-1
HQ228293	FSU10430	AY843192.1	634	Fungi sp.	4	2	0	2	623	1.00E-178	*Sarcinomyces*-1
HQ228304	FSU10585	AY843192.1	636	Fungi sp.	4	2	0	2	625	3.00E-179	*Sarcinomyces*-1
HQ228305		AY843192.1	636	Fungi sp.	4	2	0	2	619	3.00E-179	*Sarcinomyces*-1
FR773359	FSU10584	AJ292393.1	708	*Simplicillium lamellicola*	10	6	4	0	686	0	*Simplicillium lamellicola*-1
FR773268		AM992154.1	828	*Sirococcus* aff. *conigenus*	10	6	2	2	787	0	*Sirococcus conigenus*-1
FR773280	FSU8565	FM172748.1	800	Fungi sp.	10	7	1	2	769	0	*Sirococcus conigenus*-1
FR871185		FM172748.1	813	Fungi sp.	10	7	1	2	782	0	*Sirococcus conigenus*-1
HQ228294	FSU10388	GQ153162.1	802	Sordariomycetes sp.	9	8	0	1	765	0	Sordariomycetes-1
FR773234		GQ153122.1	889	Dothideomycetes sp.	50	31	10	9	856	0	*Sydowia*-1
FR773293	FSU8645	GQ412728.1	948	*Sydowia polyspora*	50	33	10	7	909	0	*Sydowia*-1
FR773346	FSU10199	AY465454.1	931	*Hormonema* sp.	50	31	11	8	889	0	*Sydowia*-1
FR773409	FSU10425	GQ153122.1	880	Dothideomycetes sp.	50	32	10	8	846	0	*Sydowia*-2
FR773294	FSU8612	FJ000374.1	821	*Tetracladium setigerum*	50	38	0	12	793	0	*Tetracladium*-1
HQ228250		EU113211.1	968	Fungi sp.	19	13	0	6	920	0	*Umbelopsis ramanniana*-1
HQ228331	FSU10293	EU715662.1	965	*Umbelopsis ramanniana*	15	9	0	6	918	0	*Umbelopsis ramanniana*-1
HQ228341	FSU10294	EU715662.1	977	*Umbelopsis ramanniana*	10	5	0	5	931	0	*Umbelopsis ramanniana*-1
FR773500	FSU10217	EU113211.1	981	Fungi sp.	13	7	0	6	933	0	*Umbelopsis ramanniana*-1
FR773285	FSU8546	EF029203.1	880	*Helicoon fuscosporum*	4	2	1	0	850	0	*Venturia*-1
HQ228256		EU434823.1	612	*Acephala* sp.	50	39	1	10	590	6.00E-172	Vibrisseaceae-1
HQ228257		EU434823.1	588	*Acephala* sp.	50	38	5	7	566	1.00E-164	Vibrisseaceae-1
HQ228274	FSU8550	EU434823.1	608	*Acephala* sp.	50	36	5	9	590	8.00E-171	Vibrisseaceae-1
HQ228276		EU434823.1	603	*Acephala* sp.	50	37	5	8	579	4.00E-169	Vibrisseaceae-1
FR773372		EU434823.1	617	*Acephala* sp.	50	37	1	12	588	1.00E-173	Vibrisseaceae-1

FR773373	**FSU8605**	**EU434823.1**	**627**	***Acephala* sp.**	**50**	**32**	**1**	**17**	**597**	**2.00E-176**	**Vibrisseaceae-1**
FR773453	**FSU10202**	**EU434823.1**	**619**	***Acephala* sp.**	**50**	**37**	**1**	**12**	**593**	**4.00E-174**	**Vibrisseaceae-1**
HQ228288		**EU434823.1**	**610**	***Acephala* sp.**	**49**	**37**	**1**	**11**	**582**	**2.00E-171**	**Vibrisseaceae-1**

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ORIGINAL ARTICLE

Comparative analyses of sooty mould communities from Brazil and Central Europe

Fabienne Flessa[1] · Janno Harjes[1] · Marcela E. S. Cáceres[2] · Gerhard Rambold[1]

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Abstract
To gain an insight into fungal sooty mould communities on leaves of trees and shrubs in the tropics and in temperate regions, 47 biofilms of the Mata Atlântica rainforest relic and the Caatinga vegetation in the state of Sergipe, Northeast Brazil, and from Central European colline and alpine zones were compared. The four sampling sites clearly differed in composition of their epiphyllous fungal communities. The fungal OTUs from all sites belonged mainly to the Ascomycota, with Dothideomycetes being the dominant class. The core community group consisted of a few site-specific representatives in co-occurrence with the ubiquitous *Mycosphaerella tassiana* and *Aureobasidium pullulans*. Most species of the core community were dark pigmented and were accompanied by facultative unpigmented or lightly pigmented species. Among the cultivable fungal species, the proportion of melanised species was significantly more abundant in samples from the two European sites, which supports the theory of thermal melanism. The identity of the host plant had a stronger impact on fungal community composition than the presence of sap-feeding insects.

Keywords Fungal community composition · Sooty mould fungi · Fungal nutrition type · Hyphal pigmentation · Mycogeography · Scale insects

Introduction

Surfaces of higher plants, particularly leaves and stems of trees and shrubs, are often colonized by dark pigmented fungi, which form hyphal mats. This type of colonization phenomenon is commonly referred to as the 'sooty mould communities' (Chomnunti et al. 2014; Kirk et al. 2010; Schoulties 1980), and can be observed in many habitats throughout the world. The absence of specific interbiotic cellular structures such as haustorial protrusions as present in truly phytopathogenic fungi (An et al. 2006; Dekhuijzen and Scheer 1969) indicates that these fungi have a predominantly or temporarily non-biotrophic life habit.

Sooty moulds are relatively common in tropical, subtropical, and warm temperate regions (Chomnunti et al. 2014). In the tropics, these (Faull et al. 2002; Olejnik et al. 1999) and other fungal associations exhibit considerable species diversity (Chomnunti et al. 2014; Dhami et al. 2013; Nelson 2008). The abundance of sooty moulds is highest during the rainy season (Batista and Ciferri 1963). Sooty moulds also occur in the cold and temperate climate zones of the European Alps, where they are formed by fungal communities with lower species numbers (Flessa and Rambold 2013).

A considerable number of sooty mould species show a clear preference for honeydew exudates of insects (Hughes 1976; Parbery and Brown 1986; Perez et al. 2009), such as aphids, whiteflies, soft scales, mealybugs, leafhoppers, and psyllids (Barr 1987; Chomnunti et al. 2014; Hamid and Jalaluddin 2006). Globally, more than 200 pigmented epiphyllous fungal species with members of the Antennulariellaceae, Capnodiaceae, Chaetothyriacae, Coccodiniaceae, Euantennariaceae, Metacapnodiaceae, and Trichomeriaceae have been recognised as belonging to this nutritional type. In their natural habitats, they may be accompanied by accessory, non-pigmented fungi (Chomnunti et al. 2014; Flessa et al. 2012; Flessa and Rambold 2013). Sooty mould fungi therefore form mixed associations with considerable proportions of pigmented species. In tropical environments, members of this community may further associate with

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✉ Gerhard Rambold
gerhard.rambold@uni-bayreuth.ch

[1] Dept. of Mycology, University of Bayreuth, 95447 Bayreuth, Germany

[2] Departamento de Biociências, Universidade Federal de Sergipe, Itabaiana, Sergipe 49500-000, Brazil

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representatives of the so-called black mildews, which belong to the Meliolaceae (Hansford 1961; Parbery and Brown 1986; Schoulties 1980) and are known as true plant parasites that develop intracellular haustoria in cells of host plants (Rodríguez and Piepenbring 2007). These obligate biotrophs interact closely with living plants to grow and reproduce, and are assumed to be host-specific (Hansford 1961; Hosagoudar 1996). Sooty mould communities are exposed to the entire airborne spore pool. Many spores can be deposited on leaves without being able to live there and an older community may therefore contain many spores that can be detected but do not contribute to the structure of the community.

To investigate the alpha diversity (Whittaker 1960) of sooty mould fungi in different continents and contrasting habitats, two sites in Brazil and two sites in Central Europe were analysed comparatively. The Brazilian sites are located in the state of Sergipe and are represented by two vegetation types. One is an open shrub vegetation with scattered trees ('Caatinga'), which exhibit typical sooty mould infestations in combination with scale insects, and the other is a predominantly secondary Mata Atlântica relic rainforest. The two Central European sites represent open habitats in the colline (Germany) and alpine (Switzerland) zones.

The aim of the present study was to test the following hypotheses: (1) The diversity of sooty mould communities is higher in the tropical region than in the central European region and also varies among the individual geographical subregions. (2) The presence of sap-feeding insects (scale insects or aphids) supports the development of specifically composed communities. (3) The community composition is also triggered by other biotic site factors such as host plant species identity. (4) The structure of the communities at all sites is similar in that the predominant fungi are dark pigmented and saprotrophic.

Material and methods

In order to investigate the differences in α-diversity of the sooty mould community at the study sites and in the two regions Central Europe and Brazil, community barcoding (CB) was performed. Since HTS does not distinguish between living and dead fungi and does not allow for a direct investigation of pigmentation, the results were complemented by a cultivation-based single-species barcoding (SB) approach. In addition, the cultivation-based approach can exclude strict biotrophs.

Sampling and conceptual design of the study

A total of 47 samples were examined with community barcoding (CB) and 33 samples with single-species barcoding (SB). In order to compare samples from ecologically distinct habitats, two sites in the neotropics (North-eastern Brazil) and two sites in the temperate zone (Central Europe) were included in this study. The two sites in the Brazilian state of Sergipe are located at lower altitudes but have two different vegetation types: Site 1 (16 samples for CB, 10 samples for SB): North-eastern Brazil Caatinga (*BC*): Brazil. Sergipe, Areia Branca, Park dos Falcões, 40 km NW of Aracaju, 7 km SE of Itabaiana, c. 7 km in north-easterly direction from Areia Branca on the road BR235 from Aracaju to Itabaiana, beside rough road at entrance of the park. Open habitat with scattered trees or shrubs. Fungi from sap-feeding insects were also studied and are labelled *BC** (4 samples for CB). Site 2 (16 samples for CB, 10 samples for SB): Northeast Brazilian Tropical Rainforest (*BR*): Brazil. Sergipe, Areia Branca, Park National Serra de Itabaiana, 36 km NW of Aracaju, 10 km SE of Itabaiana, 3 km northeast of Areia Branca on road BR235 from Aracaju to Itabaiana, beside rough road through the park area. Relic forest (Mata Atlântica rainforest) with tall trees and shrubs. Site 2 is located at a linear distance of 4 km from site 1. The two sites in Central Europe mainly differ in altitude and corresponding vegetation type: Site 3 (6 samples for CB, 8 samples for SB): Central Europe alpine zone (*EA*): Switzerland. Canton Bern, Grimsel Valley, on *Rhododendron ferrugineum* shrubs (none with sap-feeding insects). For a further characterisation of the sampling sites, see Flessa and Rambold (2013). Site 4 (5 samples for CB, 5 samples for SB): Europe colline zone (*EC*): Germany. Upper Franconia, Bayreuth, campus area and Ecological-Botanical Garden of the University of Bayreuth. For more details on the sampling sites, see Flessa et al. (2012).

Host plant sampling data are included in Supplementary Data S1. Host plants of sites BC and BR were assigned to their respective host plant identity groups based on DNA sequence analysis data. The protocol is referred in the "DNA isolation" and "PCR and sequencing" sections. The species identities of the host plants *Rhododendron ferrugineum* L. and *Salix* spp. from sites EA and EC were confirmed or revised based on morphological traits. The nomenclatural and classification concepts for the host plants follow the Catalogue of Life Annual Checklist 2019 (Roskov et al. 2019).

Associated sap-feeding insect identification: The presence or absence of the insects was recorded during the sampling period. If the insects were still on the plant during collection, they were examined microscopically and determined by morphological traits, and if amplicons of the mitochondrial cytochrome oxidase I (COI) gene were obtainable, individuals were assigned to their respective taxon. The protocol is referred to in the "DNA isolation" and "PCR and sequencing" sections.

The average annual temperature in Brazil (Itabaiana) is 25.4 °C, which differs from the European colline site with 7.8 °C and the European alpine site with 1.9 °C (climatic data resources: https://de.climate-data.org/suedamerika/brasilien/

sergipe/itabaiana-42970/; http://www.bayceer.uni-bayreuth.de/meteo/de/klima/gru/html.php?id_obj=139937; https://tirolatlas.uibk.ac.at/graphics/lieth/diag.py/chart?id=1370932).

Hypotheses 1–3 were tested primarily using the CB approach, while the SB approach was used for testing the supplementary hypothesis. Diversity indices, NMDS and PERMANOVA, were used to investigate the influence of the four sites, the two continents, the occurrence of sap-feeding insects, and the host plant family and order on the composition and diversity of the sooty mould communities. At the BC site, the sooty mould community on leaves (BC) was additionally compared with the insect-associated fungi (BC*). Overlaps and differences in the species spectrum are shown using Venn diagrams; the dominant species spectrum is shown using bar plots and a heatmap. For the most common ascomycetes from both approaches, the nutrition type was determined with FUNGuild, compared with literature, and the result visualized with iTOL Tree. To determine the proportion of dark pigmented saprotrophs, the pigmentation was examined for the most common fungi of the SB approach.

Sampling dates and sample treatment

For culture-independent community barcoding, material was collected at the four sites in October 2013. Fragments of mycelia were cut from infected leaf surfaces in the field with tweezers and scalpel, placed in 1-ml screw cap tubes, and brought to the laboratory. The tubes were kept at a temperature of ± 0 °C until DNA isolation which took place within a few days.

For species barcoding, biofilms of sooty mould communities from site EC were sampled from surfaces of young twigs of five species of *Salix* species in May 2006, as the host plants have annual leaves (Flessa et al. 2012). Biofilms of EA sooty mould communities were collected from visibly infested leaves in August 2007 (Flessa and Rambold 2013). Host plants with sooty mould communities on leaves at the BC and BR sites were sampled in October 2009. Branches of the plants were cut off, individually packed in vouchers, and transported to the laboratory. Cultivation took place immediately afterwards within a few days.

Cultivation and pigmentation test

Mycelial fragments were washed in sterile tap water, and transferred to Petri dishes containing malt-yeast medium (MYA) with 4 g glucose, 10 g malt extract, 4 g yeast extract, 0.1% tetracycline, and 12 g agar per litre. Cultures from EC, BR, and BC were incubated at room temperature and examined daily for mycelial growth. As the fungi of EA samples grew at temperatures of 15 °C or below, the incubation temperature for the fungal strains was set to 15 °C (Flessa and Rambold 2013). Outgrowing hyphae were transferred to new plates until pure cultures were obtained. Pure cultures were sorted according to their phenotypic traits (i.e. presence and type of aerial mycelia, growth form, and pigmentation type). From all isolated strains, at least one representative of each morphotype was selected for sequencing. One fungal strain per operational taxonomic unit (OTU) with more than two members and an assortment of singletons was deposited in the Jena Microbial Resource Collection (JMRC Jena). The accession numbers of the deposited strains are in Supplementary Data S2.

Pigmentation tests: Fungi were classified by their hyphal pigmentation using cultures grown on malt-yeast agar medium (MYA). Fungal cultures from EA were incubated at 15 °C and cultures from EC, BR, and BC at room temperature and examined after 1 month for macroscopically recognisable pigmentation. The pictures were taken with a Nikon D2x and lens Micro-Nikkor 60 mm F/2.8 D, with a distance of 36.5 cm to the object. The lighting was performed with two neon lamps (L18 W/19 Daylight 5000 de Luxe), which were attached to a frame with 80° inclination and provided constant lighting of the images. A colour chart (RAL 7005-HR mousy; German Institute for Quality Assurance and Certification e. V.) was used as the standard background. Settings: lens aperture of 1/16, sensitivity ISO 100, balanced preset d-0, colour mode III (Adobe RGB), and shutter speed of 0.5 s (Pietrowski et al. 2010). The CMYK values of the digital images for testing the degree of pigmentation were classified as 'dark': C: 39–73%; M: 50–75%; Y: 51–94%; K: 21–83%. CMYK values for pigmentation classified as 'light' or 'absent': C: 28–49%; M: 32–48%; Y: 33–61%; K: 0–10% according to Flessa and Rambold (2013).

DNA isolation

DNA from fungi, host plants (from BC and BR), and insects was isolated using the Charge Switch® gDNA Plant Kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA). Cell disruption was performed using a Fast Prep FP120 (Bio101, Thermo Fisher Scientific Inc., Waltham, MA, USA) with a mixture of glass beads, consisting of 0.3 g of beads with a size of 0.1 mm, 0.5 g with a size 0.5 mm, and three glass beads with a size of 2.8 mm (Peqlab) at a speed of 6.0 m s^{-1} for 2 × 40 s.

PCR and sequencing

For community barcoding, the amplification steps during the preparation of the internal transcribed spacer (ITS) library preparation for the Illumina sequencing included a two-step PCR using the ITS1F/ITS4 forward and reverse primers (Gardes and Bruns 1993; White et al. 1990) combined with specific tag sequence combinations (TAG) and the Illumina

sequencing primer to amplify the ITS1 and ITS2 regions of the rDNA gene (Supplementary Data S3a). The first PCR step was performed using 30 cycles of denaturation at 94 °C for 27 s, annealing at 57 °C for 1 s, and extension at 72 °C for 90 s. The second PCR step was performed after a purification step using Exonuclease I, with an activity of 2 U/ml (New England Biolabs), and Shrimp Alkaline Phosphatase, with an activity of 0.1 U/ml (New England Biolabs), and included 5 cycles of denaturation at 94 °C for 27 s, annealing at 53 °C for 1 s, and extension at 72 °C for 90 s, using the Illumina sequencing primer, combined with a second tag combination (INDEX) and the Illumina P5 or P7 sequencing adapters, respectively (Supplementary Data S3b). All PCR steps were performed using a Primus 96 plus thermal cycler (MWG Biotech, Ebersberg, Germany). Final amplicons were purified, quantified by gel staining with ethidium bromide in a 0.8% agarose gel and 100% UV light under a GelDoc Station (MWG Biotech), and finally pooled over several steps to minimize variations in total amplicon concentrations between samples. Paired-end sequencing was performed using an Illumina MiSeq® sequencer at the Ludwig Maximilian University of Munich Biocenter (Illumina Inc., 2018). For the list of used primers, see Supplementary Data S3.

For species barcoding, host plants, and insects, PCR was performed using MWG Biotech Primus 96 plus thermocycler. Using the Charge Switch® PCR Clean-Up Kit (Invitrogen), the obtained double-stranded sequences of the fungal ITS rRNA gene were purified and further processed according to Triebel et al. (2005) using primers ITS4 (White et al. 1990) and ITS1F-Z (5′-CTWGGYYATTTAGAGGAAGTAA; modified after Gardes and Bruns 1993). Amplicons of the host plant RNA polymerase C1 gene (rpoC1) fragment were obtained from 5 μl purified PCR products (20–80 ng/μl) with 5 μl of primers (5 pmol/μl) by GATC Biotech, by Sanger sequencing using primers rpoC1-4r (5′-CCA TAA GCA TAT CTT GAG TTG G) and rpoC1-2F (5′-GGC AAA GAG GGA AGA TTT CG) (Hollingsworth et al. 2009; Saddhe et al. 2017). Amplicons of the insect COI fragment (Folmer et al. 1994) were obtained from 5 μl purified PCR products (20–80 ng/μl) with 5 μl of primers (5 pmol/μl) by GATC Biotech using Nancy and Ron primers (Simon et al. 1994). DNA reference sequences were obtained by Sanger sequencing; consensus sequences were deposited at NCBI GenBank under accession numbers GU942834–GU942923 (fungal ITS), KM259875–KM259899 (host plant rpoC1 from BC and BR), and MN604915-MN604921 (insect COI).

Bioinformatics

For community barcoding, the reads obtained from Illumina sequencing were quality-checked using FastQC (Andrews 2010) and demultiplexed to sample-level by the variable TAG sequences, with all reads containing ambiguous base-calls removed using the pipeline Quantitative Insights Into Microbial Ecology ver. 1 (QIIME1) (Caporaso et al. 2010). Demultiplexed reads were then imported into QIIME ver. 2 (QIIME2) (Bolyen et al. 2019) and trimmed using the 'cutadapt' plugin (Martin 2011), thereby eliminating any remaining Illumina barcodes and primer sequences. To obtain amplicon sequence variants (ASVs) (Callahan et al. 2017), the DADA2 denoiser was applied as a QIIME2 plugin for dereplication, removal of all detected chimeric sequences, and quality-filtering, at a maximum expected error rate of 2 (Callahan et al. 2016). Those ASVs were further processed into OTUs (de novo) at a 97% similarity by passing their representative sequences to the VSEARCH tool, embedded in the QIIME2 pipeline (Ezeokoli et al. 2020; Rognes et al. 2016). OTU clustering was followed by an additional chimeric filtering step, applying the UCHIME implementation using the VSEARCH tool. Contextual data concerning the project and the sampling were uploaded to the NCBI databases BioProject and BioSamples. Illumina sequencing results were deposited at the NCBI 'Sequence Read Archive' (SRA) database; the uploaded demultiplexed sequences are available via the respective link from the BioProject entry with the accession number PRJNA699866. For species barcoding, fungal isolates were grouped for further statistical analysis based on their ITS rRNA sequence similarities as described by Peršoh et al. (2010). Pairwise similarities among length-adjusted sequences were calculated by using the BLAST application 'blastall' (v. 2.2.18). The resulting tabular output dataset was transformed with the simMatrix function, using the RFLPtools package (Flessa et al. 2010) of R (R, Core Team 2020). Based on this dataset, cluster analysis was performed in R with the function 'hclust' using the clustering method 'average linkage', and clusters with minimal similarities of 97% (Flessa et al. 2012) were grouped into species barcoding operational taxonomic units. The occurrence of fungal groups was noted for the samples of each host plant, yielding the present/absent matrix for further statistical analysis.

Taxonomy assignment of fungal OTUs and strains

Using the UNITE database as a reference, a naïve Bayesian classifier was trained on the UNITE v.8.0 dynamic dataset (Nilsson et al. 2019) and used to assign the taxonomy to fungal OTUs via the QIIME2 pipeline applying the command *feature-classifier classify-sklearn* with a confidence threshold of 70% required for taxonomy assignment.

Statistical data analysis

The frequency table, its taxonomy, and metadata (in which the geographic site and occurrence of leaf-sucking insects as well

as the phylogenetic affiliation of the host plant taxon were encoded as grouping factors) of community and species barcoding approaches were converted to CSV format, imported in R v.3.6.2 (R Core Team 2020), and subsequently merged to an S4 object using 'phyloseq' v1.30.0 (McMurdie and Holmes 2013). Unless otherwise stated, all evaluations and analyses were performed with R. Plots of rarefaction curves were built with the 'ggrare' function in the 'ranacapa' package v. 0.1.0 (Kandlikar 2019). A dissimilarity matrix on relative abundance was calculated using the dissimilarity method most appropriate for the data obtained with the 'rankindex' function in the 'vegan' v.2.5–6 package (Oksanen et al. 2019), i.e. Manhattan dissimilarities. Variation of fungal communities between samples for community barcoding was visualized by non-metric multidimensional scaling (NMDS) in the 'vegan' package using the 'metaMDS' function and plotted with package 'ggplot2' v.3.2.1 (Wickham 2016). For the species barcoding dataset, Jaccard similarity was calculated and NMDS was visualized in Primer 6 (v. 6.1.11, Primer-E Ltd., UK). Permutational multivariate analysis of variance (PERMANOVA) was performed on both approaches with the 'adonis' function in the 'vegan' package to compare fungal communities based on the factor sampling site, continent, sap-feeding insect occurrence, host plant family, and order. To perform pairwise comparisons between individual factors, pairwise PERMANOVA was conducted using the package 'pairwiseAdonis' v.0.0.3 (Martinez 2019) for significance testing ($p < 0.05$) applying Benjamini-Hochberg false discovery rate adjustments on all p values to protect against false positives (Benjamini and Hochberg 1995; Ricks and Koide 2019). It was assumed that the p value indicated significant differences if it was less than 0.05. The 'adonis' function calculates an R value that indicates the extent to which the factors analysed explain the groupings. R values were interpreted as follows: > 0.75 indicating different groups, $R > 0.5$ overlapping different ones, and $R < 0.25$ indicating slightly separate groups.

Venn diagrams were drawn using 'VennDiagram' v.1.6.0 (Chen 2018). Alpha diversity indices, i.e. Chao1 richness estimate, Shannon-Wiener diversity index, Simpson's dominance, and Pielou's evenness, were calculated with the 'alpha' function in the package 'microbiome' v1.8.0 (Lathi and Shetty 2017) and plotted with 'ggplot2'. Bar plots of phylum, class, and order were drawn using the 'tax_glom' function in the package 'phyloseq' and plotted with package 'ggplot2'. Heatmaps were plotted from all OTUs, which were present in at least 10% of all samples using the package 'pheatmap' v.1.0.12 (Kolde 2019) and 'RColorBrewer' v.1.1–2 (Neuwirth 2014). All analyses described in this section were performed on all OTUs without any further selection; heatmaps were based on those OTUs, which were present in at least 10% of all samples.

Fungal guild nutrition type of the predominant Ascomycota

From the OTUs belonging to the Ascomycota, those that were present in at least 3 samples and had a taxonomic assignment at genus or species level and a guild assignment at species, genus, or family level were selected using the FUNGuild database (Nguyen et al. 2016). If there was no entry in the database, the nutrition type as recorded in the Dictionary of Fungi was used (Kirk et al. 2010). If there was no entry in either source, OTUs from community barcoding were classified as 'n.a.' (not assignable). For strains from species barcoding, mixotrophic or eventually saprotrophic nutrition type was assigned due to their cultivability. In addition to the rough classifications into saprotrophic, mixotrophic, and biotrophic nutrition types, the following life habit subtypes were applied: P: plant-associated (including litter saprotrophic and wood saprotrophic); A: animal-associated; S: saprotrophic; and O: 'other' (i.e. non-specific, fungus-associated, lichen-associated, dung saprotrophic); U: non-specific saprotrophic. The representative sequences were aligned using Mega7 software (Kumar et al. 2016) and a maximum likelihood phylogenetic tree (Egidi et al. 2019) was built using a Kimura 2-parameter model. For the visualization, the tree was uploaded to iTOL V5.5 (Letunic and Bork 2019) and supplemented with the nutrition type information and taxonomic classification.

Results

Dataset descriptions

Sooty mould symptoms were formed by oligo-species communities with an average of 11.2 species per biofilm sample from culture-based species barcoding and of 26.7 OTUs per sample from culture-independent community barcoding. Single-species communities are almost non-existent. In both approaches, > 60% of all OTUs could be assigned at order level or higher, and about one-third at species level. The proportion of OTUs that could not be assigned at all is below 26%. The rarefaction curves generated for each sample showed saturated curves for the community barcoding approach (Supplementary Data S4.1a). For a summary of the number of OTUs and their occurrence as well as a list of taxa detected by one or both approaches, see Supplementary Data S5.

Impact of the factors site and continent (community barcoding)

Differences in alpha diversity indices: The average expected number of OTUs per sample is highest in North-eastern Brazil Caatinga (BC) and the Europe Alpine (EA) zone, and lowest

in scale insects from BC (BC*), whereas the difference in the number of species between the samples of BC* is the lowest, and the greatest in the Central European colline (EC) zone (Fig. 1a). The expected number of OTUs per sample is similar in both continents. The Shannon-Wiener index shows differences in the diversity of OTUs at all sites, with the two European sites (EA and EC) clearly differing from the Brazilian ones (Fig. 1a). Less OTUs dominate in Brazilian samples than in European ones, as the two European sites have considerably lower dominance values than the sites in South America, with EC samples differing more markedly from the others. European colline (EC) and Brazil rainforest (BR) show a higher degree of evenness in the composition of their fungal communities than Brazil Caatinga (BC) and Europe Alpine (EA) (Fig. 1a). While 5 OTUs are shared by all sites, another 6 occur at three sites each. BC and BR have the highest number of exclusively shared OTUs among all pairs, with 30 OTUs. However, most OTUs can only be found in samples of one site (from 75 in EA to 184 in EC). Thirty-seven OTUs occur in samples on both continents (Supplementary Data S4.2). The NMDS on fungal community similarity showed that BR samples are the most widely dispersed (Fig. 2), the two European sampling sites are clearly separated, and EC is close to BC and shows overlap with BR, but this overlap is due to a single sample of BR with few reads. At a threshold of 95%, all sampling sites overlap. PERMANOVA yielded significant results ($p = 0.001$) for differences in community composition between sites and continents (Table 1). However, as the r^2 value of the factor 'continent' ($r^2 = 0.101$) is very low, it explains the differences in community composition less well than the factor 'site'. The pairwise PERMANOVA between the groups of factor 'site' showed significant results between all site pairs (Table 1) for both approaches.

Table 1 (A) PERMANOVA analysis of differences in the community compositions among factors site, continent, sap-feeding insect—association, taxonomic affiliation of the host plants, and combined factors. (B) Pairwise PERMANOVA of the factor 'site'. North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC)

(A) PERMANOVA: Global		
Factors	r^2	p
Site	0.347	0.001
Continent	0.101	0.001
Sap-feeding insect presence	0.092	0.001
Host plant family	0.465	0.001
Host plant order	0.308	0.001
Host plant family and sap-feeding insect presence	0.577	0.001
Host plant order and sap-feeding insect presence	0.396	0.001
(B) PERMANOVA: Pairwise, factor 'site'		
Pairs	r^2	p*
BC vs BC*	0.083	0.094
BC vs BR	0.178	0.002
BC vs EC	0.283	0.002
BC vs EA	0.394	0.002
BC* vs BR	0.121	0.002
BC* vs EC	0.331	0.089
BC* vs EA	0.576	0.005
BR vs EC	0.139	0.002
BR vs EA	0.212	0.002
EC vs EA	0.590	0.005

*Benjamini-Hochberg false discovery rate adjustments to protect pairwise PERMANOVA against false positives

Impact of sap-feeding insects (community barcoding)

Based on the comparison of the community composition on what is the main nutritional source provided by the habitat, three types of sources could be distinguished: exudates by secretion from leaf glands, substances by leaching from leaves, and exudates from leaf-sucking insects. While leaching occurs to some extent on every host plant, glandular secretion was confirmed only for the EA host plant. Traces or remains of sap-feeding insects were confirmed in the field for leaves of host plants from BC. In EC, traces or remains of sap-feeding insects (Aphididae) were observed on all sampled trees in the field. No leaf-sucking insects or traces thereof were found at BR and EA sites. The grouping factor 'sap-feeding insect association' leads to a similar number of expected OTUs per sample, similar diversity, and only slightly different dominance and evenness between the two groups (Fig. 1c). Of all OTUs, 58 occur in both groups (Supplementary Data S4.2). Differences in community composition under the factor 'sap-feeding insect association' showed a significant impact ($p = 0.001$) in the PERMANOVA. However, as the r^2 value of this factor ($r^2 = 0.092$) was very low, it explains the differences in community composition less well than other factors. For the BC site, samples of sooty moulds from leaves and fungal OTUs from sap-feeding insects on infected plants (labelled as BC*) were compared. The communities found on and in sap-feeding insects showed complete overlap with those of leaf samples and proximity to the EC samples in the NMDS analysis. The pairwise PERMANOVA between groups of the factor 'site' showed significant results between all pairs except between BC and BC* and BC* and EC (Table 1), which is also consistent with the NMDS analysis.

Host plant dependence (community barcoding)

PERMANOVA revealed significant differences ($p = 0.001$) in community composition between host plant families and orders (Table 1). Significant differences were found in the

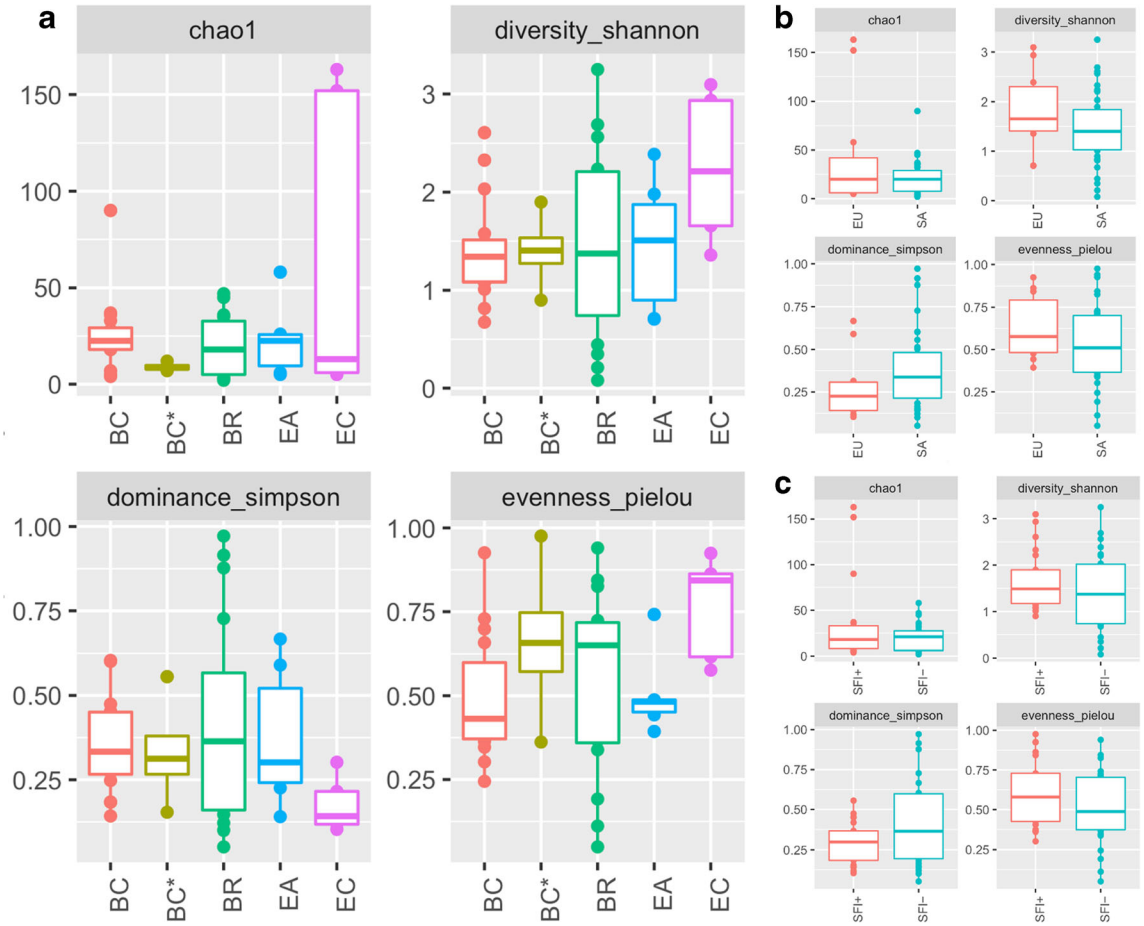

Fig. 1 Alpha diversity estimates of fungal communities **a** among the sites North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC). **b** Continents Europe (EU) and South America (SA). **c** Association with sap-feeding insects (SFI+ = sap-feeding insect presence, SFI− = no sap-feeding insect association)

pairwise comparisons between 9 host plant orders. The corresponding table of results is given in Supplementary Data S6, and the pairwise comparisons of host plant families in Supplementary Data S7. Sooty mould communities could not be found on all plants of a site, and the host plant families and orders were strongly linked to the sites, i.e. Asterales and Dilleniales occurred only at BC, Ericales only at EA, and Gentianales and Sapindales only at BR. This factor should therefore be viewed with some caution. The fungal community compositions differ significantly ($r^2 = 0.304$, $p = 0.003$) when 'host plant order' and 'site' are tested as a combined factor, but there are no significant results in the pairwise tests (data not shown). The community compositions are also significantly separated based on the paired factor 'host plant order' or 'host plant family' with 'sap-feeding insect association' (Table 1). Although the global PERMANOVA gives a high r^2 value for host plant family and sap-feeding insect, none of the pairwise PERMANOVA tests is significant (data not shown). Pairwise tests of host plant order and sap-feeding insect between 9 groups were significant. The corresponding table of results is given as Supplementary Data S6.

Taxonomy (community barcoding)

While OTUs were used anonymously for all previous analyses, the analyses in the following section are based on taxonomic assignments. Ascomycota and Basidiomycota were found at all sites, with Ascomycota predominating (Fig. 3a). Within the Ascomycota, Dothideomycetes were the dominant class at all sites, followed by Eurotiomycetes and Sordariomycetes, which were present at all four sites (Fig. 3b). Within the Dothideomycetes, the Capnodiales and Pleosporales dominated and were also present at all sites. Capnodiales are the dominant order, with a proportion of over 50% in BC incl. BC* and EA, over 25% in EC, and over 10% in BR, considering the high proportion of unassignable ones in BR. Both European sites are characterised by a higher relative abundance of Eurotiomycetes. Among the Sordariomycetes, the Hypocreales and Xylariales were the dominant order, but were mainly found in samples from BC and less frequent in BR. The Xylariales were absent in the group 'sap-feeding insects' (BC*) and were found in EA and EC in only one sample each. Within Basidiomycota,

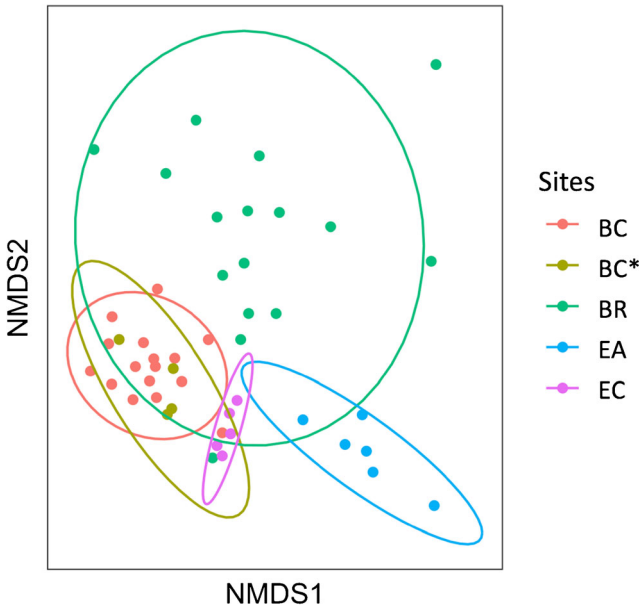

Fig. 2 Non-metric multidimensional scaling (NMDS) ordination plot showing the compositional differences of the sooty mould communities from the different sampling sites North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC). Confidence interval of ellipses = 95%, Manhattan dissimilarities, stress = 0.158

Tremellomycetes were predominant and were found at all sites (Fig. 3c) but their relative abundance is higher at BC and EC sites than at the other sites. BR samples showed a higher relative abundance of unassignable OTUs (NA). The steadiness of OTUs that were present in at least 10% of all samples is summarized in Fig. 4. The Brazilian sites differ from the two European sites in community structure. However, the two European sites are less similar than the Brazilian ones. *Mycosphaerella tassiana*, *Aureobasidium pullulans*, and *Vishniacozyma* sp. Pleosporales sp. occurred in BC, BR, EA, and EC. Capnodiaceae sp. and *Capnodium* sp. occurred only at the Brazilian sites, and Capnodiales sp. 3 and *Sarcinomyces* sp. only in EA samples. Capnodiaceae sp. and *Capnodium* sp. tend to co-occur. This also applies to *Sarocladium strictum* and *Alternaria* sp. together with *Erythrobasidium hasegawaianum* and Capnodiales sp. with *Sarcinomyces* sp.

In order to classify the trophic mode within the Ascomycota via FUNGuild, those OTUs were selected that were present in at least three samples and that had a taxonomic assignment to species or genus level. The criteria applied to 29 OTUs, representing 4.27% of all OTUs in this dataset and 37.17% of all reads, which were combined in an iTOL tree (Fig. 5). Thirteen of these OTUs have a biotrophic trophic mode, 9 are classified as saprotrophic, and another 7 are declared mixotrophic according to FUNGuild. Three-quarters of OTUs are plant-associated, 5 of them are also animal-associated. All those belonging to the saprotrophic type were classified as unspecific. None of the OTUs was exclusively animal-associated.

Single-species community barcoding

The complementary analyses of cultivable fungi showed similar results to the community barcoding in terms of the influence of the factors 'site', 'continent', 'sap-feeding insect association', and 'host plant family and order'. All factors examined were significant. Again, the r^2 values for the grouping factor 'continent' (r^2 = 0.163) and sap-feeding insect presence (r^2 = 0.09) were lower than those of the other factors (Supplementary Data S8.2a). In contrast to community barcoding, BC and BR samples overlap completely in the NMDS, while EA and EC are separate and do not overlap with Brazil samples (Supplementary Data S8.1, 8.3c–5c). Pairwise PERMANOVA between site factor groups showed significant results between all pairs except for the two Brazilian ones (Supplementary Data S8.2b), which is consistent with NMDS analysis. The cultivable part of the European communities is represented by a much lower number of species per sample than of the South American ones. One species was found in all four sites, most (37) are shared by BC and BR (Supplementary Data Fig. S8.4b). Ascomycota and Basidiomycota were found at all sites, with Ascomycota predominating (Supplementary Data Fig. S8.6a). The ratio Ascomycota to Basidiomycota is similar to that of community barcoding. The order Capnodiales within the Dothideomycetes is also the group with the highest relative abundance (Supplementary Data Fig. S8.6b–c). Likewise, also the Sordariomycetes show a higher abundance in BC and BR. Among the orders, the relative proportion of Pleosporales is considerably higher at all sites. This also applies to Xylariales in the BR and BC samples (Supplementary Data Fig. S8.6b–c). In contrast to community barcoding, only *Aureobasidium pullulans* occurs at all four sites; *Mycosphaerella tassiana* and *Didymella exigua* occur in a large number of samples from BC, BR, and EC, and *Antennariella placitae* mainly in BC samples and to a lesser extent in BR and EA samples. In accordance with the results obtained with community barcoding, Capnodiales sp. was found exclusively at the EA site and there in 85% of the samples (Supplementary Data Fig. S8.7). Trophic modes according to FUNGuild for the most common Ascomycota are given in Supplementary Data S8.8.

Pigmentation of sooty mould fungi

The degree of pigmentation was determined by taking into account the steadiness of the species. The proportion

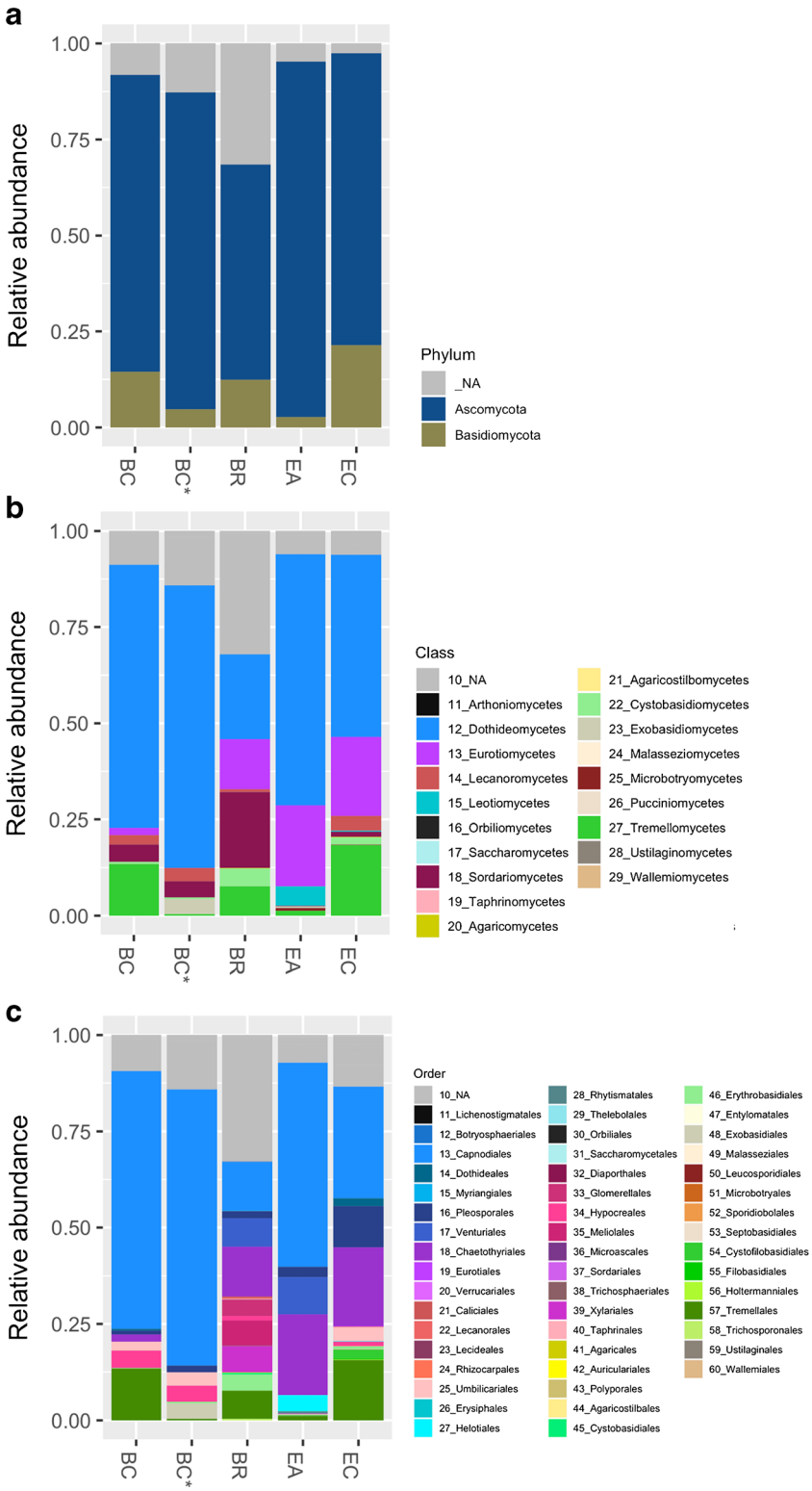

Fig. 3 Relative abundance of **a** phyla (Ascomycota and Basidiomycota), **b** class (Ascomycota: 11–19, Basidiomycota: 20–29), and **c** order (11: Arthoniomycetes; 12–17: Dothideomycetes; 18–20: Eurotiomycetes; 21–25: Lecanoromycetes; 26–29: Leotiomycetes; 30: Orbiliomycetes; 31: Saccharomycetes; 32–39: Sordariomycetes; 40: Taphrinomycetes; 41–43: Agaricomycetes; 44: Agaricostilbomycetes; 45–46: Cystobasidiomycetes; 47–48: Exobasidiomycetes; 49: Malasseziomycetes; 50–52: Microbotryomycetes; 53: Pucciniomycetes; 54–58: Tremellomycetes; 59: Ustilaginomycetes; 60: Wallemiomycetes) in North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC) samples

of detections of dark pigmented fungal species was 46.2% across all fungal species. Of those present in at least one-third of all samples, 60% were dark pigmented, and of those present in at least half of all samples, 100% were dark pigmented. This analysis shows that the more common fungi at each site were dark pigmented. At all four sites, the proportion of dark pigmented species varied widely, with the highest value in EC (60%) and lower values in BC (47.5%) and EA (46.3%), and lowest in BR (43.8%). Among the predominant which were present

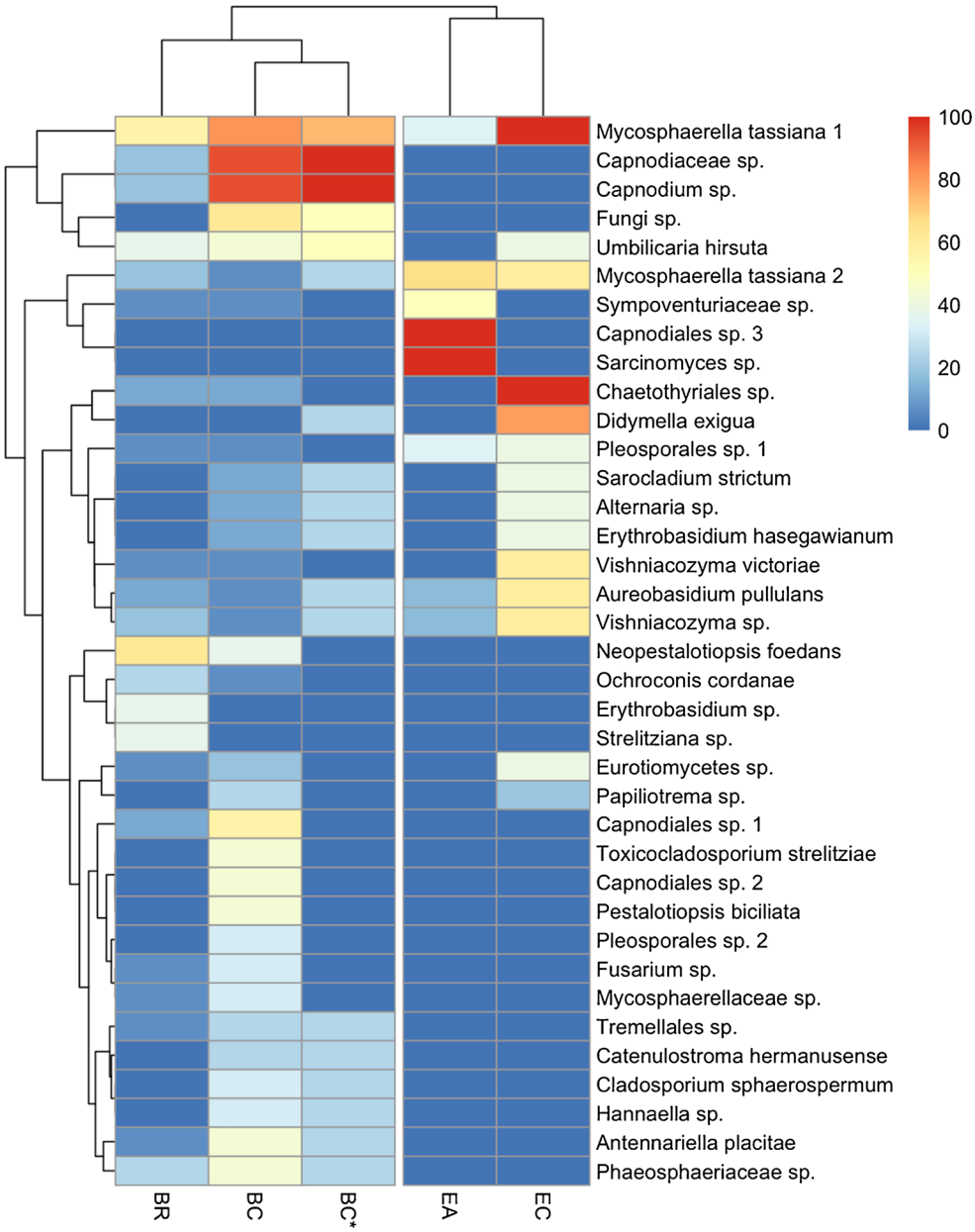

Fig. 4 Heatmap of the most common taxa based on their consistency in North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC) samples. The colour scale illustrates the dominance (%) within the sites. The dendrogram on top illustrates the similarity of the sites; the one on the left represents the similarity in the pattern of occurrence of the individual fungal OTUs

in at least one-third of all samples, the ratio shifts towards the dark pigmented ones with the highest values in EA (100%) and EC (67%) and slightly lower proportion in BC (59%) and lowest value in BR (48%). A trend for a correlation between nutrition type and pigmentation could not be found (Fig. 6) neither generally for all fungi nor for those found in at least one-third of the samples per site. The dark pigmented fungi of this study could all be assigned to Ascomycota, while all Basidiomycota were light pigmented (Supplementary Data Fig. S8.9). Dothideomycetes were the largest class among the dark pigmented and Sordariomycetes represented the largest group among the light pigmented ones.

Discussion

Applied methods

This study used a cultivation-based species barcoding approach (SB) and a community barcoding approach (CB). With regard to the significant impact of the factors site, continent, sap-feeding insect presence, and host plant family and order on fungal community composition, the results for both approaches are similar. For community barcoding, the ITS1 region was used as marker. In recent studies, the use of the two ITS regions has been quite inconsistent. While some used ITS2 as the universal fungal barcode (Bao et al. 2019; Beng

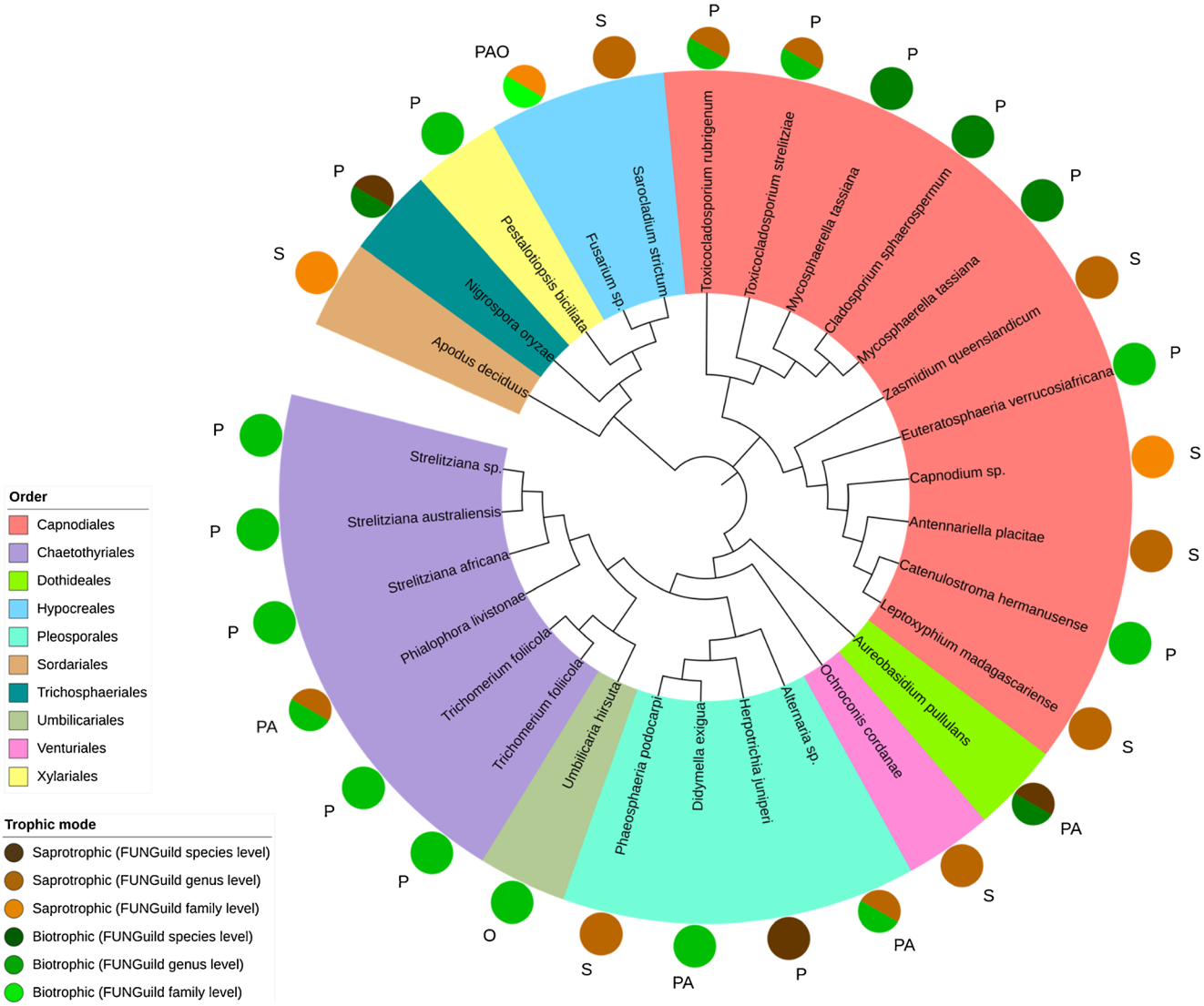

Fig. 5 Most abundant OTUs (occur in at least 3 samples) among the Ascomycota with taxonomic assignment on species or genus level and with FUNGuild classification on species, genus, or family level. Tree was generated with maximum likelihood method by Mega7 and visualized with iTOL (interactive Tree of Life). P, plant-associated (including litter saprotrophic and wood saprotrophic); A, animal-associated; S, saprotrophic; O, 'other' (i.e. unspecific, fungus-associated, lichen-associated, dung saprotrophic)

and Corlett 2019; Egidi et al. 2019; Epstein et al. 2019; Li et al. 2019; Lynikiene et al. 2020; Morales-Rodriguez et al. 2019; Nerva et al. 2019; Qian et al. 2020; Ricks and Koide 2019; Saravesi et al. 2019; Wilkinson et al. 2019; Würth et al. 2019; Yao et al. 2019; Zhang et al. 2019), others strongly recommended the use of ITS1 (Mbareche et al. 2020), as it had already been implemented in many studies (Beule et al. 2019; Checinska Sielaff et al. 2019; Del Frari et al. 2019; Schiro et al. 2019). Few studies are based on both ITS regions (Janakiev et al. 2019). The use of operational taxonomic units (OTUs) versus the recent use of amplicon sequence variants (ASVs) is also inconsistent. Although there are recommendations to use only ASVs (Callahan et al. 2017), resulting in a reduction in the number of dominant species (Egidi et al. 2019), many of the current studies are still based on OTUs (Bao et al. 2019; Beng and Corlett 2019; Beule et al. 2019; Checinska Sielaff et al. 2019; Epstein et al. 2019; Janakiev et al. 2019; Lynikiene et al. 2020; Morales-Rodriguez et al. 2019; Nerva et al. 2019; Pan et al. 2019; Qian et al. 2020; Ricks and Koide 2019; Saravesi et al. 2019; Wilkinson et al. 2019; Würth et al. 2019; Yao et al. 2019; Zhang et al. 2019), but others rely on ASVs (Del Frari et al. 2019; Egidi et al. 2019; Li et al. 2019), or OTUs generated from ASVs (Ezeokoli et al. 2020), and even OTUs from cultivation-based Sanger sequencing are still in use (Janowsky et al. 2019). With the availability of the VSEARCH tool within the QIIME2 pipeline, allowing the easy aggregation of OTUs from ASVs, the Illumina sequencing datasets in this

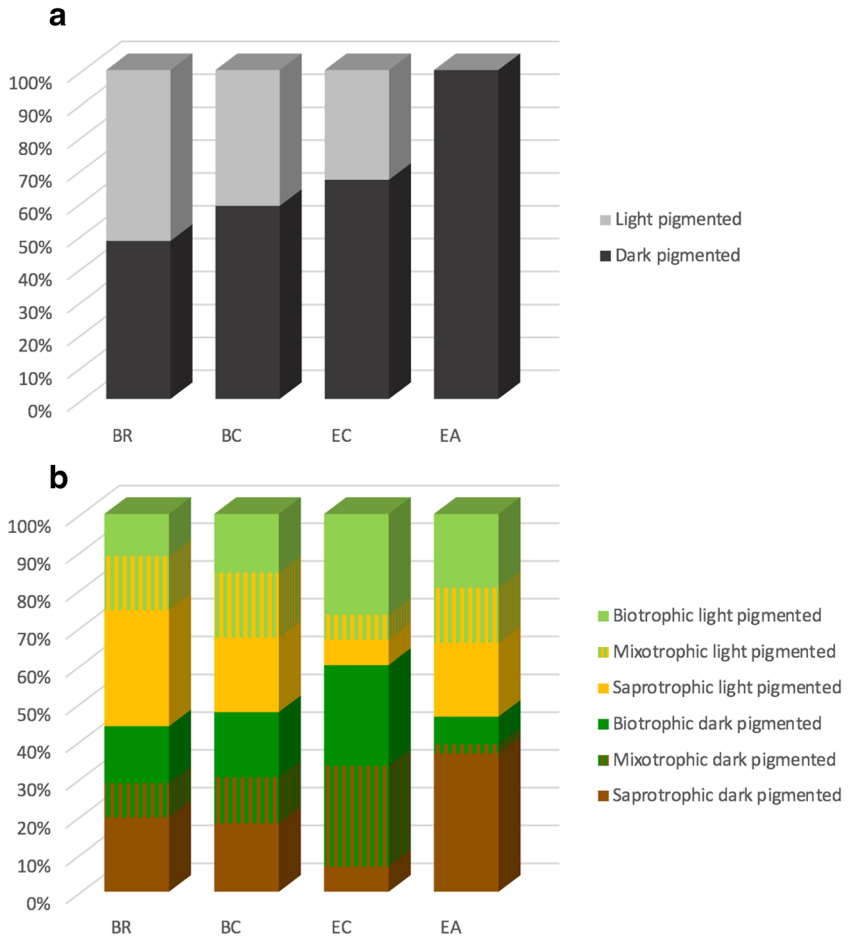

Fig. 6 Proportion of pigmented species and link between pigmentation and nutrition type. **a** Proportion of dominant pigmented species (present in at least 1/3 of all samples at this site) taking into account their steadiness. North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA), and Central Europe colline zone (EC). **b** All species regardless of their dominance from species barcoding are grouped by pigmentation and nutrition types based on the relative amounts of samples they were found in

study were analysed based on ASVs, obtained from the DADA2 plugin from QIIME2, with subsequent grouping into OTUs, based on a sequence similarity threshold.

By including a culture-based method, it was possible to include the degree of pigmentation of the mycelia as an analytical factor in the design of this study, to detect the fungi that are mixotrophic and to exclude those which are exclusively biotrophic. Those aspects cannot be addressed by the exclusive use of a culture-independent approach. Also, the fungi found with the different approaches may differ (Dissanayake et al. 2018). To put the significance of the pigmentation tests into perspective, it must be taken into account that pH, carbon, and nitrogen source have a major influence on pigment production in fungal strains (Lathadevi et al. 2014). Therefore, pigmentation may differ in culture and in situ. It is well documented that the degree of pigmentation of fungal cultures depends on the medium (Kowalski et al. 2016; Palacio-Barrera et al. 2019; Blechert et al. 2019). In a previous study of sooty mould fungi associated with *R. ferrugineum*, fewer fungi were dark pigmented on a MYA medium than on other media (Flessa and Rambold 2013). However, any fungus that was dark pigmented on MYA was also dark pigmented on the other media. It can therefore be assumed that the proportion of dark pigmented fungi of sooty mould communities in situ is possibly underestimated.

Host plants of tropical parasitic black mildews (Meliolaceae) in Brazil are known to belong to Anacardiaceae, Asteraceae, Euphorbiaceae, and Fabaceae (Hansford 1961; Hosagoudar 1996; Macedo et al. 2010; Pinho et al. 2012; Pinho et al. 2009; Silva et al. 2012). These host plant families overlap with the host spectrum of sooty mould fungi in the present study. Black mildews have similar traits as the pigmented saprotrophic species, such as the degree of pigmentation and hyphal growth, and can coexist within sooty mould communities. Due to their biotrophic habit, efforts to cultivate black mildews have not been successful (Hansford 1961; Hosagoudar 1996) and they are therefore not included in the species barcoding approach of this study.

At all four sites, sampling efforts were driven by the discovery of dark pigmented epiphytic communities on perennial plant substrates, regardless from the identity of the host plant species. In an earlier study of sooty mould communities on the evergreen *Rhododendron ferrugineum*, differences in the composition between communities on young leaves and those on previous years leaves were recognised (Flessa and

Rambold 2013). A similar effect was observed for the community on annual leaves and perennial structures of *Salix* spp. in a previous study, and on hibernating evergreens in a greenhouse (Flessa et al. 2012). In the current study design, therefore, only perennial plant parts were compared for all four sites. In the comparative study on sooty mould communities on *Salix* and evergreen plants in the same region, the influence of different climatic annual cycles was analysed. The sooty mould communities on perennial plant parts of both host groups differed less than the sooty mould communities on annual and perennial structures within one group. As seasonal aspects may have less influence on the composition of sooty mould fungi than on other organisms (Flessa et al. 2012), the chosen study design is suitable to address the hypotheses of the present study. Since sooty moulds were only found on hosts of one genus per site at both European locations, but at the Brazilian sites on host plants of different orders and families, conclusions about host plant dependence can be drawn at least for the two sites in Brazil.

Most current studies do not distinguish between epiphytic and endophytic fungal communities, as the complete microbiome is usually extracted from whole leaves (Lynikiene et al. 2020; Qian et al. 2020; Würth et al. 2019). This is problematic in that endophytic fungi are more host-dependent than epiphytic fungi (Fonseca-García et al. 2016; Lau et al. 2013; Peršoh 2013). Furthermore, it has been shown that the two microbiomes differ considerably and only share a small proportion of the strains (Flessa and Rambold 2013; Yao et al. 2019). In this study, therefore, a methodological approach was chosen that allows to focus on epiphytic communities.

Differences in community composition between continents and sampling sites

The significant difference between the alpha diversity of European and Brazilian sites reflects the distance between the two phytogeographical zones, which differ in climatic conditions and vegetation types. The significant difference in the sooty mould communities can therefore be explained to a considerable extent by climatic differences. In Brazil, the vegetation cycle is usually interrupted by a long dry season with a rise in temperature due to the lack of rain. This has a strong influence on all organisms, comparable to the strong influence of seasonal effects on communities at European sites, but also plants in Europe may be exposed to severe drought in summer and winter (Aulitzky et al. 1982; Cernusca 1976; Rango and Martinec 1994; Sakai and Larcher 1987). However, since the grouping factor 'continent' explains the different sooty mould communities less well than the individual sampling sites, the large-scale climatic differences are not the main factor. One possible reason for this overlap could be the dry periods which occur in both sites in Brazil and Europe and may be a stronger driving factor than different average temperatures. Another reason could be that there are generally many ubiquitous fungi in the sooty mould community, and indeed, 5 OTUs occur at all sites.

The different sites (BC, BR, EA, EC) influenced the community composition significantly, with much less overlap between groups. Since all four sites were close to busy roads, the differences cannot be explained by different levels of pollution. Although both European sampling sites are located in the temperate zone, there are significant differences in the type of vegetation surrounding the sampling sites and the mean annual temperature due to the altitude. Climate data from the Botanical Garden weather station at EC showed that the highest amounts of rainfall coincided with the highest temperatures in July and August and with the lowest temperatures in December and January. At EA, the highest amount of precipitation corresponds to the month with the lowest mean temperatures (Tirol Atlas, Geographie Innsbruck). Thus, since the Brazilian fungal communities from Caatinga and rainforest are located at a short distance apart, macroclimatic factors cannot explain these differences. This finding contrasts with the results of Dhami et al. (2013), who found no differences in community composition between two more distant sampling sites with different host plant species, when using a community barcoding approach. In this study, host influence was tested only between plant individuals of two species of the same genus (*Nothofagus*), but not at higher taxonomic levels. Maybe the differences between two species within a genus are too small to have an influence on the community composition.

Influence of sap-feeding insect association on community composition

There is evidence from many studies that sooty moulds have relationships with leaf-sucking insects because they are able to grow on honeydew (Chomnunti et al. 2014; Perez et al. 2009). However, the presence of honeydew alone does not lead to a defined community composition because significant differences could be observed between communities of EC and BC. Climate and vegetation type are therefore most important, but probably also the fact that honeydew composition greatly differs between Aphididae (EC) and Coccidae (BC). The dominant sugar components in the honeydew of *Aphis fabae* (Aphididae) are the trisaccharide melezitose and fructose (Fischer et al. 2005), while in *Coccus hesperidum* (Coccidae), glucose and sucrose are the main sugar components (Golan and Nadja 2011). Different types of sugars (mono-, di-, and trisaccharides) as additional nutrition sources to the substances leached from the leaf tissues could therefore have an indirect influence on the composition of the fungal community. However, the most abundant OTUs were found on plants of both Brazilian sites, although sap-feeding insects were only found on BC host plants. This indicates that the

occurrence of those genera is not strictly dependent on the presence of scale insects and honeydew as a nutrition source. Nevertheless, other fungi may be strongly affected by the presence or absence of honeydew in addition to substances leached from the leaf tissues (Tukey 1970). Based on the results of this study, it can be assumed that, although honeydew provides additional carbohydrates for the sooty mould community, it does not lead to a specific sooty mould community as an isolated factor. As the fungi found in sap-feeding insect samples (BC*) largely overlap with those in the sooty mould community on leaves of the same site, these insects could play a role in the dispersal of sooty moulds within a region. It is possible that only a part of the fungi is spread by insects, and others, such as Xylariales, by airborne spores. However, this requires further investigation.

Host-species selectivity

The host plants studied in the tropics and the host plant *Rhododendron ferrugineum* in the European Alps have perennial leaves. On the European colline site (with mostly deciduous trees), 'saturated' epifoliar fungal communities were found on branch tips of *Salix* spp. and were sampled from there. Thus, at all four sampling sites, premature termination of succession in fungal communities due to leaf fall as a consequence of seasonality can be excluded (Flessa et al. 2012). Host plant order and family had a significant impact on the community composition in both approaches. Differences in the structure of the fungal community among host plant species can be caused by differences in surface structure and nutritional status on leaf surfaces. Leaching substances, which may persist of most compounds found in plants, may serve as other sources than honeydew (Tukey 1970) and vary between different host plant species. This widespread process of leaching leads to large nutrient losses (Schoch 1955; Wallace 1930), so that leaching substances can easily accumulate on the leaf surface of one plant species, but may be swept away from others due to different surface structures.

The assumption that host plant taxonomy has a great influence on phyllosphere fungal community composition is consistent with the study by Kembel and Mueller (2014), who showed that in the tropics the taxonomic identity of the host plant explains more than half of the variation of the composition of fungal communities across trees. The result of the present study is not fully conclusive, however, because of the 15 pairwise tests that included orders from the tropical sites, only 5 were significantly different and among the families only one. This result is not surprising, however, as pathogenic and endophytic fungi are known to be more restricted to certain host plant genera or species, while saprotrophs, which dominate the communities described here, are less host-specific (Kodsueb et al. 2008; Zhou 2001). A large proportion of cultivable fungal species in this study were described as mixotrophic. This two-track nutrition type may be responsible for this relatively low degree of selectivity between host plants. Although host plant order alone does not explain the differences well, five significant results of the pairwise tests of the combined factors 'host plant order' and 'sap-feeding insect association' from the Brazil sites at least suggest that different leaching in relation to honeydew may have an influence on community composition. However, this would need to be verified in more detail in future studies. Communities on Ericales and Ericaceae from EA differed significantly from orders and families of the other sites. Presence of fungi in the EA samples was mostly restricted to the undersides of the leaves. Leachates or glandular secretions, as present on leaves of *Rhododendron ferrugineum* (Flessa and Rambold 2013), may provide a substrate leading to a more specific sooty mould community. However, as sooty moulds occurred exclusively on *R. ferrugineum* in the alpine study site, this cannot be conclusively assessed.

Fungal community structure: taxonomic groups, pigmentation, and nutrition types

The fact that a numerous coexisting species compete for the same resource requires more attention. According to the niche theory, the most common result is competitive exclusion, and it is to be expected that single-species should dominate over oligo-species communities (Tubay et al. 2015). This was observed in *Rhododendron*-associated fungal communities, where glandular secretions in young leaves were correlated with the dominant occurrence of a sooty mould fungus (Flessa and Rambold 2013). However, this fungus is not able to suppress the growth of other fungi. On perennial leaves, the predominance of that fungal species decreased towards pre- or co-dominance with a ubiquitous species. This result is similar to the situation on evergreen trees sampled in the colline zone (Flessa et al. 2012). The high proportion of oligo-species communities in older *Rhododendron ferrugineum* leaves, on stems of *Salix* spp., and on the various leaves of tropical trees thus suggests that, according to the niche theory, more traits added by the community-inhabiting fungi themselves lead to more complex and different niches, which in turn can be filled with additional fungal species.

Capnodiales, common fungi in all four habitats studied, are usually dark pigmented. Capnodiaceae are sooty moulds in the strict sense and their dominance in Brazil is consistent with previous studies on tropical sooty moulds. Together with species of Antennulariellaceae, Capnodiaceae, Chaetothyriaceae, Coccodiniaceae, Euantennariaceae, and Metacapnodiaceae, they dominate the saprotrophic epifoliar fungal community forming sooty mould symptoms (Chomnunti et al. 2014; Dhami et al. 2013; Faull et al. 2002; Olejnik et al. 1999). However, they do not occur in such communities in Europe. There, this niche is occupied by Chaetothyriales sp. (EC) and

Capnodiales sp. in coexistence with *Sarcinomyces* sp. (EA). Of the four OTUs that were ubiquitous at all sites, only *Mycosphaerella tassiana* (dark pigmented, mixotrophic according to FUNGuild and its cultivability) was also among the more common fungi at all four sites. Less continuous, but present at all sites was *Aureobasidium pullulans* (mixotrophic, light and dark pigmented).

Fungi which were present in at least two-thirds of all biofilms form the core community of the sooty mould symptom. It is formed by specific, non-ubiquitous and a few ubiquitous fungi. The OTUs, which were present in less than 1/3 of all samples, are considered sporadic companions rather than members of the main matrix in the biofilm of the sooty mould community. A large proportion of these are light pigmented. For this group of fungi, the absence of dark pigmentation on leaf surfaces may be a disadvantage under natural conditions as discussed below. Causes for this lack of pigmentation may also be in vitro effects caused by the type of cultivation media (Lee and Hyde 2002, Lathadevi et al. 2014) so that the proportion of dark pigmented ones could be even higher. Among the common fungi, the proportion of dark pigmented species is lowest in the warmest regions and highest in the colder regions. This is in accordance with the thermal melanism theory (Clusella Trullas et al. 2007) which states that dark pigmented ectothermal organisms have an advantage at low temperatures because they can warm up more easily. Saprotrophic fungi have been subject of a large-scale study in Europe and were found to be darker in cold environments (Krah et al. 2019), which supported the theory of thermal melanism and has now also been confirmed for sooty mould fungi in this study: the proportion of dark pigmented predominant species is lowest in the warmest regions BR (48%) and BC (59%) and higher in the colder regions EC (67%) and EA (100%). Sooty mould communities comprise primary pigmented fungi and accessory unpigmented fungi. The main group consists of functional species that are dark pigmented. Melanin protects against high UV radiation (Zak 2005) and chemical and biological stress, leads to desiccation resistance and structural re-enforcement of hyphal walls (Butler and Day 1998; Eisenman and Casadevall 2012), and leads to the advantage of faster warming in cold regions (Krah et al. 2019). Unpigmented fungi lacking this trait may be considered accessory endomycelial immigrants. A correlation of dark pigmentation with a saprotrophic or mixotrophic nutrition strategy and light pigmentation with a biotrophic strategy was not observed, based on the results of the single-species barcoding approach. It is assumed that both dark pigmented and less or unpigmented species of this fungal community can also live saprotrophically. Some of them may optionally be biotrophic or fungicolous, which is also likely for dark pigmented ones (Butler et al. 2001). In their epifoliar life stage, unpigmented fungi may be dependent on the primary group. The incrustations formed by dark pigmented fungi are thus heterospecific mycelia consisting of primary and secondary fungal components. Biofilms of bacteria of different compositions and structures are well known (Davey and O'Toole 2000; Stahl et al. 2013). They mostly involve semi-aquatic accumulations of prokaryotes. However, there is no reason not to consider heterospecific mycelia as a type of biofilm, according to the definition by Characklis (1990), which refers to 'cells at a substratum, a surface accumulation, which is not uniform in time or space, a significant amount of inorganic or abiotic substances held together by the biotic matrix and transport and transfer processes play[ing] a much more important role than in isolated occurrence of suspended single cells' (Characklis 1990). Another indication for different structural niches within the epifoliar fungal community are the different nutritional types. Most species and OTUs (of both approaches) are totally or at least partially saprotrophic. Only 1/5 of all OTUs in this study were identified as biotrophic according to the FUNGuild classification. However, this result has to be put into perspective in that species from the culture-based approach that are classified as biotrophic may only be facultatively biotrophic due to their cultivability. The proportion of possible saprotrophs may be underestimated if the FUNGuild reference alone is used to determine the nutritional type. Of the biotrophic or potentially biotrophic OTUs, the largest proportion was plant-associated with only a small proportion classified as potentially fungicolous.

Conclusions

Based on the results of this study, the core community of sooty moulds can be characterised as follows: Only few species generally dominate and form a main group. Among them, there are usually one or few specific representatives that occur together with a few ubiquitous species with dark pigmentation. Other unpigmented or weakly pigmented fungi are considered accessory and are saprotrophic or biotrophic, the latter interacting directly with their hosts. Their presence depends on the presence of main group representatives that form a protecting structure. In this context, the theory of thermal melanism was supported for the first time for sooty mould communities. Diversity and composition of sooty mould communities depend on site and climatic conditions as well as on the identity of the host plant, but to a lesser extent on the presence of sap-feeding insects. We propose to subsume the heterospecific mycelial complex of sooty mould communities under the term 'biofilm'. In this study, a first impression could be gained about the potential of comparative mycogeographic studies on habitat and host plant preferences of sooty moulds.

Further studies are needed to investigate the influence of sap-feeding insects in more detail.

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Author contribution Fabienne Flessa co-designed the project, collected the environmental samples from Europe, carried out part of the laboratory work as well as all the statistical analyses in this study, and wrote the original draft of the manuscript taking into account the input of the co-authors. Janno Harjes wrote sections on methodology and bioinformatics, carried out part of the laboratory work, and established and run the bioinformatic pipeline for community barcoding. Marcela Cáceres collected the environmental samples from Brazil. Gerhard Rambold designed and supervised the project, revised the manuscript, and collected and provided the environmental samples from Brazil.

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Availability of data and materials Sequences from single-species barcoding are available in the NCBI GenBank under accession numbers GU942834–GU942923, KM259875–KM259899, and MN604915-MN604921. Sequences from community barcoding are linked under BioProject accession number PRJNA699866. Reference strains of the single-species community barcoding were deposited in the Jena Microbial Resource Collection (JMRC Jena) as specified under "Material and methods". Further data cited in the manuscript are available as Supplementary Data. All other relevant data is available upon request.

Declarations

Ethics approval This article does not contain any studies with human or animal subjects

Conflict of interest The authors declare no competing interests.

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S1 Host plant collection site details and GenBank accession numbers

Community barcoding

Sooty mould communities on leaves of different branches of the same plant are marked a and b

Host Plant Coll. No.	Collection Site Latitude and Longitude	Site Altitude	Taxon Name	GenBank Accession No.
BC-2013-01a	S10°44'53.58" W37°22'39.54"	312 m	Chrysobalanaceae-1	KM259895
BC-2013-01b	S10°44'53.58" W37°22'39.54"	312 m	Chrysobalanaceae-1	KM259895
BC-2013-02a	"	"	Asteraceae-1	KM259893
BC-2013-03a	"	"	Asteraceae-1	KM259896
BC-2013-03b	"	"	Asteraceae-1	KM259896
BC-2013-04a	"	"	Asteraceae-1	KM259888
BC-2013-04b	"	"	Asteraceae-1	KM259888
BC-2013-05a	"	"	Bunchosia-1	KM259885
BC-2013-05b	"	"	Bunchosia-1	KM259885
BC-2013-07a	S10°44'54.96" W37°22'38.34"	310 m	Asteraceae-1	KM259889
BC-2013-07b	S10°44'54.96" W37°22'38.34"	310 m	Asteraceae-1	KM259889
BC-2013-08a	S10°44'53.46" W37°22'37.2"	318 m	*Dillenia indica*-1	KM259878
BC-2013-08b	S10°44'53.46" W37°22'37.2"	318 m	*Dillenia indica*-1	KM259878
BC-2013-09a	S10°44'53.46" W37°22'37.2"	318 m	*Hymenolobium flavum*-1	KM259897
BC-2013-09b	S10°44'53.46" W37°22'37.2"	318 m	*Hymenolobium flavum*-1	KM259897
BC-2013-10a	S10°44'53.46" W37°22'37.2"	318 m	*Dillenia indica*-1	KM259879
BC*-2013-03	S10°44'53.58" W37°22'39.54"	"	Asteraceae-1	KM259896
BC*-2013-05	S10°44'53.58" W37°22'39.54"	"	Bunchosia-1	KM259885
BC*-2013-08	S10°44'53.46" W37°22'37.2"	318 m	*Dillenia indica*-1	KM259878
BC*-2013-10	S10°44'53.46" W37°22'37.2"	318 m	*Dillenia indica*-1	KM259879
BR-2013-12a	S10°45'46.38" W37°20'24.48"	197 m	Euphorbiaceae-1	KM259890
BR-2013-12b	S10°45'46.38" W37°20'24.48"	197 m	Euphorbiaceae-1	KM259890
BR-2013-13a	S10°45'45.96" W37°20'23.94"	198 m	*Hirtella*-1	KM259875
BR-2013-14a	S10°45'45.42" W37°20'30.3"	209 m	*Hirtella*-1	KM259877
BR-2013-14b	S10°45'45.42" W37°20'30.3"	209 m	*Hirtella*-1	KM259877
BR-2013-17a	S10°45'36.54" W37°20'22.5"	206 m	*Bunchosia*-1	KM259883
BR-2013-18a	"	"	*Bunchosia*-1	KM259884
BR-2013-18b	"	"	*Bunchosia*-1	KM259884
BR-2013-19a	S10°49'35.16" W37°20'21.9"	"	Anacardiaceae-1	KM259898
BR-2013-20a	"	"	*Pera bumeliifolia*-1	KM259881
BR-2013-20b	"	"	*Pera bumeliifolia*-1	KM259881
BR-2013-22a	"	"	*Pera bumeliifolia*-1	KM259882
BR-2013-23a	S10°49'34.8" W37°20'21.96"	"	*Thyrsodium puberulum*-1	KM259891
BR-2013-24a	S10°45'29.1" W37°20'24.72"	207 m	Apocynaceae-1	KM259886
BR-2013-40a	S10°45'36.54" W37°20'22.5"	"	Apocynaceae-1	KM259887
BR-2013-40b	S10°45'36.54" W37°20'22.5"	"	Apocynaceae-1	KM259887
EA-2013-01a	N46°33' 41.30" E8° 20' 5.44"	2200 m	*Rhododendron ferrugineum*	n.a.
EA-2013-02a	N46°34'23.64" E8°20'11.00"	1870 m	*Rhododendron ferrugineum*	n.a.
EA-2013-03a	N46°35' 31.97" E8°19' 33.65"	1750 m	*Rhododendron ferrugineum*	n.a.
EA-2013-03b	N46°35' 31.97" E8°19' 33.65"	1750 m	*Rhododendron ferrugineum*	n.a.
EA-2013-04a	N46°35'48.50" E8°19'33.55"	1620 m	*Rhododendron ferrugineum*	n.a.
EA-2013-04b	N46°35'48.50" E8°19'33.55"	1620 m	*Rhododendron ferrugineum*	n.a.
EC-2013-01a	N49°55'32.26" E11°34'58.34"	"	*Salix nigricans*	n.a.
EC-2013-02a	"	"	*Salix apennina*	n.a.
EC-2013-03a	"	"	*Salix elaeagnos*	n.a.
EC-2013-04a	"	"	*Salix purpurea*	n.a.
EC-2013-05a	N49°55'43.11" E11°35'4.44"	"	*Salix cinerea*	n.a.

Single species barcoding

Host Plant Coll. No.	Collection Site Latitude and Longitude	Site Altitude	Taxon Name	GenBank Accession No.
BC-2009-01a	S10°44'53.9" W37°22'39.5"	302 m	Asteraceae-1	n.a.
BC-2009-01b	S10°44'53.9" W37°22'39.5"	302 m	Asteraceae-1	n.a.
BC-2009-02a	S10°44'53.6" W37°22'40.0"	"	Asteraceae-1	n.a.

BC-2009-02b	S10°44'53.6" W37°22'40.0"	"	Asteraceae-1	n.a.
BC-2009-03a	S10°44'53.7" W37°22'40.1"	"	Asteraceae-1	n.a.
BC-2009-03b	S10°44'53.7" W37°22'40.1"	"	Asteraceae-1	n.a.
BC-2009-04a	S10°44'53.5" W37°22'38.9"	"	Asteraceae-1	n.a.
BC-2009-04b	S10°44'53.5" W37°22'38.9"	"	Asteraceae-1	n.a.
BC-2009-05a	S10°44'49.5" W37°22'37.5"	311 m	Asteraceae-1	KM259892
BC-2009-05b	S10°44'49.5" W37°22'37.5"	311 m	Asteraceae-1	KM259892
BR-2009-01a	S10°45'25.2" W37°20'30.7"	253 m	*Hirtella*-1	KM259876
BR-2009-01b	S10°45'25.2" W37°20'30.7"	253 m	*Hirtella*-1	KM259876
BR-2009-02a	S10°45'30.9" W37°20'23.4"	208 m	*Dillenia*-1	KM259894
BR-2009-02b	S10°45'30.9" W37°20'23.4"	208 m	*Dillenia*-1	KM259894
BR-2009-03a	S10°45'56.7" W37°20'25.7"	340 m	Sapotaceae-1	KM259899
BR-2009-03b	S10°45'56.7" W37°20'25.7"	340 m	Sapotaceae-1	KM259899
BR-2009-04a	S10°45'36.0" W37°20'22.3"	205 m	Dilleniaceae-1	KM259892
BR-2009-04b	S10°45'36.0" W37°20'22.3"	205 m	Dilleniaceae-1	KM259892
BR-2009-05a	S10°45'36.0" W37°20'22.3"	205 m	Dilleniaceae-1	KM259892
BR-2009-05b	S10°45'36.0" W37°20'22.3"	205 m	Dilleniaceae-1	KM259892
EA-2007-01a	N46°33'43.11" E8°20'6.45"	2150 m	*Rhododendron ferrugineum*	n.a.
EA-2007-01b	N46°33'43.11" E8°20'6.45"	2150 m	*Rhododendron ferrugineum*	n.a.
EA-2007-02a	N46°34'23.64" E8°20'11.00"	1870 m	*Rhododendron ferrugineum*	n.a.
EA-2007-02b	N46°34'23.64" E8°20'11.00"	1870 m	*Rhododendron ferrugineum*	n.a.
EA-2007-03a	N46°35'32.59" E8°19'32.62"	1750 m	*Rhododendron ferrugineum*	n.a.
EA-2007-03b	N46°35'32.59" E8°19'32.62"	1750 m	*Rhododendron ferrugineum*	n.a.
EA-2007-04a	N46°35'48.50" E8°19'33.55"	1620 m	*Rhododendron ferrugineum*	n.a.
EA-2007-04b	N46°35'48.50" E8°19'33.55"	1620 m	*Rhododendron ferrugineum*	n.a.
EC-2006-01a	N49°55'32.26" E11°34'58.34"	352 m	*Salix nigricans*	n.a.
EC-2006-02a	"	"	*Salix apennina*	n.a.
EC-2006-03a	"	"	*Salix elaeagnos*	n.a.
EC-2006-04a	"	"	*Salix purpurea*	n.a.
EC-2006-05a	N49°55'43.11" E11°35'4.44"	"	*Salix cinerea*	n.a.

S2 Reference strains of the single-species community barcoding

GenBank accession number	Tax assignment	Confidence	Jena Microbial Resource Collection (JMRC) strain number
HE584856	*Mycosphaerella tassiana* 3	0.99999637	FSU10303
HE584958	*Pestalotiopsis rhododendri*	0.82702365	FSU10358
HE584837	*Antennariella placitae*	0.98044748	FSU10304
HE584972	*Neopestalotiopsis foedans*	0.99970764	FSU10299
HE585008	*Didymella exigua* 2	0.99947697	FSU10310
HE584828	*Capnodium* sp.	1	FSU10590
HE584839	Fungi sp.	1	FSU10353
HE585003	Phaeosphaeriaceae sp. 2	0.99967581	FSU10601
HE584816	*Aureobasidium pullulans*	0.99999989	FSU10296
HE584965	*Pseudopestalotiopsis theae* 2	0.99920347	FSU10356
HE584905	Botryosphaeriaceae sp.	0.99999525	FSU10302
HE584888	Nectriaceae sp.	0.92382007	FSU10308
HE584899	*Trichoderma harzianum*	0.99879315	FSU10363
FR773176	Capnodiales sp.	0.99333858	FSU8620
HE584948	*Ascochyta maackiae*	0.76811141	FSU10364
HE584874	*Cosmospora gigas*	0.99999999	FSU10357
HE584908	*Lecanicillium longisporum*	0.81815648	FSU10597
HE584879	*Coniothyrium* sp. 1	0.99994193	na
HE584945	*Nigrospora oryzae*	0.99998757	FSU10603
HE585025	*Toxicocladosporium strelitziae*	0.96572381	FSU10298
HE584951	Aspergillaceae sp.	0.99829173	FSU10297
HE584918	*Meira geulakonigii*	0.83394006	na
HE584930	Sordariomycetes sp.	0.96441868	FSU10362
HE584868	*Coniothyrium* sp. 2	0.99975396	FSU10360
HE584817	*Aureobasidium thailandense*	1	FSU10575
HE584883	Tremellales sp.	0.96965686	FSU10596
HE584880	*Erythrobasidium hasegawianum*	1	FSU10300
HE584875	*Hannaella kunmingensis*	0.89567633	FSU10301
HE584917	Pleosporales sp. 2	0.97928905	FSU10579
HE584923	*Mycosphaerella* sp.	0.99983633	na
HE584941	*Myriangium* sp.	1	na
HE585032	*Pseudozyma hubeiensis*	0.99999991	FSU10307
HE584820	*Candida amphicis*	0.96391385	FSU10311
HE584912	*Pyrenochaetopsis leptospora* 2	0.9999999	FSU10580
HE585034	Xylariaceae sp.	0.80646787	FSU10595
HE584869	*Corynespora smithii*	1	FSU10588
HE584913	*Leptoxyphium madagascariense* 1	0.99999971	FSU10572
HE584946	*Ochroconis cordanae*	1	FSU10581
HE584921	*Paraconiothyrium* sp.	0.81110498	FSU10317
HE584814	Sympoventuriaceae sp.	0.99999846	FSU10350
HE584897	Dothideomycetes sp. 1	0.77841229	FSU10589
HE584922	*Dothistroma pini*	0.99972076	FSU10587
HE584885	*Fusicolla* sp.	0.99729076	FSU10582
HE585018	*Mycotribulus mirabilis*	1	na

HE584841	*Catenulostroma hermanusense* 1	0.99575926	na
FR773178	*Mycosphaerella tassiana* 1	0.99999184	na
FR773193	*Phacidium lacerum* 2	0.99893466	na
HE584919	Montagnulaceae sp.	0.9752982	FSU10301
HE585019	Phaeosphaeriaceae sp. 1	0.99431514	FSU10351
HE584813	Pleosporaceae sp. 1	0.99999036	FSU10361
HE584867	Didymosphaeriaceae sp. 1	0.83545001	FSU10309
HE584925	Didymosphaeriaceae sp. 3	0.99981687	na
HE584911	*Pyrenochaetopsis leptospora* 1	0.99999993	na
HE585021	*Acrocalymma aquatica* 1	0.9999807	FSU10598
HE584876	*Daldinia starbaeckii*	0.99995203	FSU10314
HE585035	*Xylaria* sp.	0.98889524	na
HE585030	Hypocreaceae sp.	0.99999938	na
FR773192	*Neoascochyta paspali*	0.99999997	FSU10605
HE584939	Pleosporales sp. 1	0.96081554	FSU10594
HE584999	*Pseudopestalotiopsis theae* 1	0.99967611	FSU10593
HE584842	*Catenulostroma hermanusense* 2	0.86771847	FSU10573
FR871179	*Exobasidium nobeyamense*	0.79899577	FSU8609
HE584942	Hypomyces sp.	0.97592417	FSU10574
FR773215	*Leptosphaeria errabunda*	0.72612623	FSU8678
HE584927	*Periconia byssoides*	0.98003288	na
FR773182	*Phacidium lacerum* 1	0.99957438	na
FR773206	*Phacidium lacerum* 3	0.99883897	FSU8562
FR773218	*Seimatosporium eucalypti*	0.83955564	FSU8682
HE584934	Cucurbitariaceae sp.	0.99518703	FSU9718
FR773328	*Didymella exigua* 1	0.91162359	FSU10344
FR773189	Didymellaceae sp.	0.99888762	FSU10339
FR773213	Mycosphaerellaceae sp.	0.83058036	na
FR773315	*Peniophora* sp.	0.97658704	FSU8547
FR773302	*Alternaria metachromatica*	0.9999999	FSU6492
HE584884	Didymosphaeriaceae sp. 2	0.99982403	FSU10577
HE584866	Didymosphaeriaceae sp. 4	0.99320183	FSU18316
HE584924	*Acrocalymma aquatica* 2	0.98994075	FSU10578
FR773314	*Arbusculina fragmentans*	0.99999887	FSU8606
HE584823	Capnodiaceae sp. 2	0.99962688	FSU10306
FR773313	*Chalara holubovae*	0.99999997	FSU10420
HE584881	Erythrobasidium sp. 1	1	na
HE584882	Erythrobasidium sp. 1	1	FSU10592
FR773203	*Heterobasidion araucariae*	0.99999842	FSU8560
FR773201	*Hypholoma fasciculare*	0.99999937	FSU8619
FR773330	*Lachnellula calyciformis*	0.99997381	FSU8548
HE584926	Lasiosphaeriaceae sp.	0.99801707	na
HE584916	*Leptoxyphium madagascariense* 2	0.99999996	na
HE585022	*Ochroconis sexualis*	1	na
GU942919	*Umbelopsis gibberispora*	0.99954479	na
FR773329	*Annulohypoxylon multiforme*	1	FSU10381
HE584819	*Clonostachys rosea*	0.99999806	FSU10365
FR773212	Ascomycota sp.	0.71673001	FSU10412

HE584878	Diaporthales sp. 4	0.99999814	FSU10367
FR773202	Dothideales sp.	0.91038808	FSU10409
HE584937	Dothideomycetes sp. 2	0.71577197	FSU10591
FR773341	Dothioraceae sp.	1	na
FR773303	Fungi sp.	0.88350645	FSU10343
FR773312	Leotiomycetes sp.	0.99999966	FSU10419
FR773301	Microbotryomycetes sp.	0.99969676	FSU8568
HE585020	Pleosporales sp. 2	0.85430761	FSU10315
FR773322	Sarcinomyces sp.	0.99999987	FSU10380
HE584935	*Wojnowiciella eucalypti*	0.72430107	FSU10354
HE585036	Xylariales sp. 2	0.87272879	na

S3 Primer list community barcoding

From: Peršoh, D., Stolle, V., Brachmann, A., Begerow, D., Rambold, G., 2018. Fungal guilds are evenly distributed along a vertical spruce forest soil profile while individual fungi show pronounced niche partitioning. Myc. Prog. https://doi.org/10.1007/s11557-018-1405-6

3a Forward and reverse primer 1st PCR community barcoding library preparation (Persoh, 2018)

ITS1F_1 TACACGACGCTCTTCCGATCTTCATCTTGGTCATTTAGAGGAAGTAA
ITS1F_2 TACACGACGCTCTTCCGATCTAAGTGACTTGGTCATTTAGAGGAAGTAA
ITS1F_3 TACACGACGCTCTTCCGATCTTGCGAGACTTGGTCATTTAGAGGAAGTAA
ITS1F_4 TACACGACGCTCTTCCGATCTGACATCCACTTGGTCATTTAGAGGAAGTAA

ITS4_T1 CAGACGTGTGCTCTTCCGATCTAGGAGTCCTCCGCTTATTGATATGC
ITS4_T2 CAGACGTGTGCTCTTCCGATCTCGCTCATCCTCCGCTTATTGATATGC
ITS4_T3 CAGACGTGTGCTCTTCCGATCTGCTAACATCCTCCGCTTATTGATATGC
ITS4_T4 CAGACGTGTGCTCTTCCGATCTTTGACCAGTCCTCCGCTTATTGATATGC

3b Forward and reverse primer 2nd PCR community barcoding library preparation (Persoh, 2018)

PSP_501
AATGATACGGCGACCACCGAGATCTACACTAGATCGCACACTCTTTCCCTACACGACGCTCTTCCGATCT

PSP_502
AATGATACGGCGACCACCGAGATCTACACCTCTCTATACACTCTTTCCCTACACGACGCTCTTCCGATCT

PSP_503
AATGATACGGCGACCACCGAGATCTACACTATCCTCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

PSP_504
AATGATACGGCGACCACCGAGATCTACACAGAGTAGAACACTCTTTCCCTACACGACGCTCTTCCGATCT

PSP_505
AATGATACGGCGACCACCGAGATCTACACGTAAGGAGACACTCTTTCCCTACACGACGCTCTTCCGATCT

PSP_701
CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

PSP_702
CAAGCAGAAGACGGCATACGAGATCGTACTAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

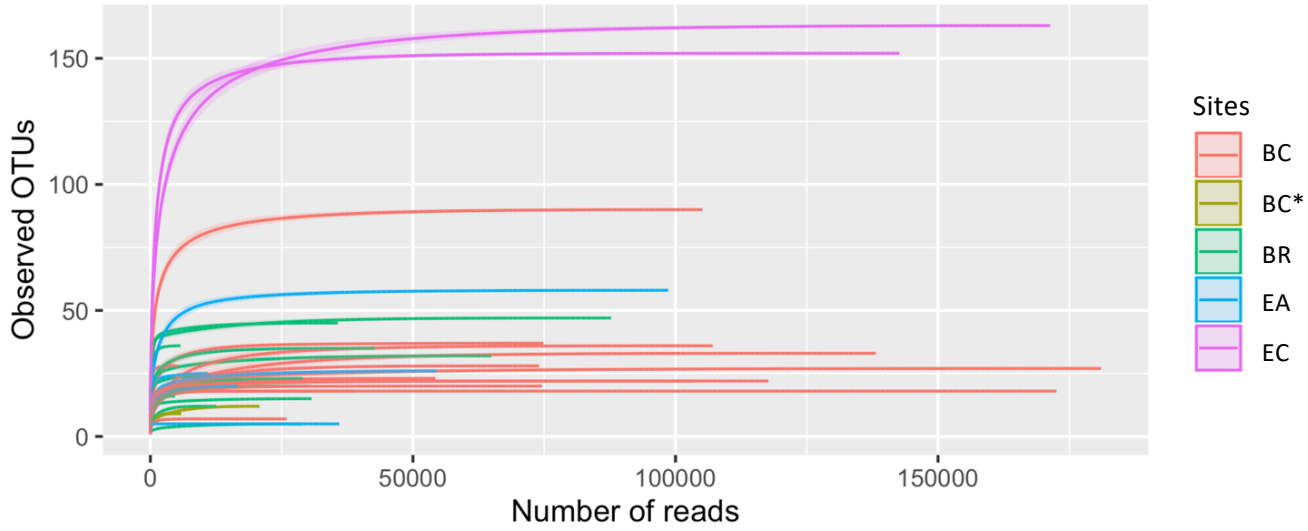
PSP_703
CAAGCAGAAGACGGCATACGAGATAGGCAGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

PSP_704
CAAGCAGAAGACGGCATACGAGATTCCTGAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

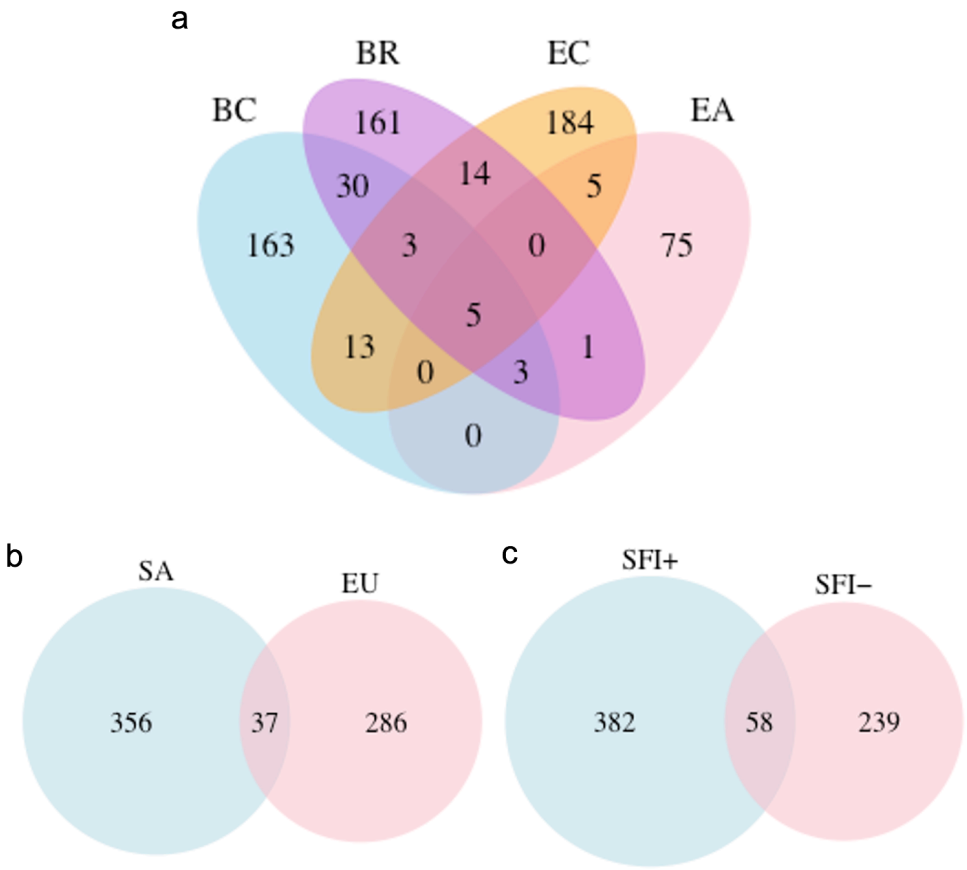
PSP_705
CAAGCAGAAGACGGCATACGAGATGGACTCCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

S4 Supplementary figures of the community barcoding approach

S4.1



S4.1 Rarefaction curves of all samples. The colour code and legend correspond to the sites from which the samples originate: North-eastern Brazil Caatinga leaves (BC) and sap-feeding insects (BC*), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA) and Central Europe colline zone (EC).



S4.2 Venn plots: Overview of shared and exclusive OTUs. a) In the four sites North-eastern Brazil Caatinga (BC, of the 163 site-specific OTUs, 117 occurred just in one sample); North-eastern Brazil Tropical Rainforest (BR, of the 161 site-specific, 138 occurred just in one sample); Europe alpine zone (EA, of the 75 site-specific, 54 occurred in just one sample); Central Europe colline zone (EC, of the 184 site-specific, 153 occurred in just one sample), b) between the two continents Europe (EU) versus South America (SA) and c) between samples with sap-feeding insect (SFI+) and without sap-feeding insect (SFI−) association.

S5 Comparison of community barcoding and single species barcoding approach

S5.1 Number of OTUs and their appearance in all samples of the study and assignability of taxonomic names. NA: Taxonomic assignment only up to kingdom level, no more precise assignment possible.

Taxonomic level	Community barcoding	Species barcoding
Total OTUs	679	104
Occurrence of OTUs	2'218'881	358*
NA [%]	25.5	1.9
Species [%]	27.8	52.4
Genus [%]	39.8	65
Family [%]	44.9	84.5
Order [%]	63.5	92.2
Class [%]	68.3	97.1
Phylum [%]	74.5	98.1
Average number of OTUs per sample	26.7	11.2
Proportion of single-OTU communities	0	1

*Occurrence was registered only as present/absent in each sample.

S5.2 Taxa detected by one or both approaches. Each taxon listed was found in at least two samples with one of the two approaches and could be assigned to the order level or lower.

	Detected by community barcoding	Detected by single species barcoding
Alternaria metachromatica	x	x
Antennariella placitae	x	x
Aureobasidium pullulans	x	x
Aureobasidium thailandense	x	x
Capnodiaceae sp.	x	x
Capnodiales sp.	x	x
Capnodium sp.	x	x
Catenulostroma hermanusense	x	x
Coniothyrium sp.	x	x
Cucurbitariaceae sp.	x	x
Didymella exigua	x	x
Didymosphaeriaceae sp.	x	x
Dothideales sp.	x	x
Dothideomycetes sp.	x	x

Erythrobasidium hasegawianum	x	x
Erythrobasidium sp.	x	x
Leotiomycetes sp.	x	x
Leptoxyphium madagascariense	x	x
Microbotryomycetes sp.	x	x
Montagnulaceae sp.	x	x
Mycosphaerella sp.	x	x
Mycosphaerella tassiana	x	x
Mycosphaerellaceae sp.	x	x
Myriangium sp.	x	x
Nectriaceae sp.	x	x
Neoascochyta paspali	x	x
Neopestalotiopsis foedans	x	x
Nigrospora oryzae	x	x
Ochroconis cordanae	x	x
Ochroconis sexualis	x	x
Paraconiothyrium sp.	x	x
Periconia byssoides	x	x
Phaeosphaeriaceae	x	x
Pleosporaceae sp.	x	x
Pleosporales sp.	x	x
Sarcinomyces sp.	x	x
Sordariomycetes sp.	x	x
Sympoventuriaceae sp.	x	x
Toxicocladosporium strelitziae	x	x
Tremellales sp.	x	x
Alternaria sp.	x	
Apodus deciduus	x	
Arthrinium pseudospegazzinii	x	
Articulospora proliferata	x	
Bannoa hahajimensis	x	
Buckleyzyma aurantiaca	x	
Bullera crocea	x	
Bullera penniseticola	x	
Chaetothyriales sp.	x	
Cladophialophora minutissima	x	
Cladosporium sphaerospermum	x	
Cryptococcus sp.	x	
Cuniculitremaceae sp.	x	
Cyberlindnera jadinii	x	
Cystobasidium pinicola	x	
Devriesia sardiniae	x	
Devriesia sp.	x	
Dioszegia cryoxerica	x	
Dioszegia hungarica	x	
Erythrobasidiales sp.	x	
Erythrobasidium elongatum	x	
Eurotiomycetes sp.	x	
Euteratosphaeria verrucosiafricana	x	

Exobasidium sp.	x	
Fellomyces sp.	x	
Filobasidium sp.	x	
Filobasidium wieringae	x	
Flagellospora fusarioides	x	
Fusarium sp.	x	
Genolevuria sp.	x	
Halosphaeriaceae sp.	x	
Hannaella luteola	x	
Hannaella siamensis	x	
Hannaella sp.	x	
Helotiales sp.	x	
Herpotrichia juniperi	x	
Herpotrichiellaceae sp.	x	
Holtermanniales sp.	x	
Hortaea werneckii	x	
Itersonilia perplexans	x	
Knufia endospora	x	
Kondoa yuccicola	x	
Lecania cyrtella	x	
Lecania naegelii	x	
Leptosphaeria rubefaciens	x	
Malassezia globosa	x	
Meira argovae	x	
Meira nashicola	x	
Meliola thailandicum	x	
Mrakiella aquatica	x	
Mycosphaerella etlingerae	x	
Neodevriesia shakazului	x	
Orbiliales sp.	x	
Papiliotrema pseudoalba	x	
Papiliotrema sp.	x	
Papiliotrema terrestris	x	
Penidiella sp.	x	
Pestalotiopsis biciliata	x	
Phaeophleospora hymenocallidicola	x	
Phaeosphaeria podocarpi	x	
Phaeosphaeriaceae sp.	x	
Phialophora livistonae	x	
Physcia dubia	x	
Physcia kalbii	x	
Plectosphaerella sp.	x	
Sarocladium strictum	x	
Sclerotiniaceae sp.	x	
Sporobolomyces oryzicola	x	
Sporobolomyces roseus	x	
Strelitziana africana	x	
Strelitziana australiensis	x	

Strelitziana sp,	x	
Taphrina inositophila	x	
Teratosphaeriaceae	x	
Teratosphaeriaceae sp.	x	
Toxicocladosporium rubrigenum	x	
Toxicocladosporium sp.	x	
Tremella roseotincta	x	
Tremellaceae sp.	x	
Trichomeriaceae sp.	x	
Trichomerium dioscoreae	x	
Trichomerium foliicola	x	
Umbilicaria hirsuta	x	
Venturiaceae sp.	x	
Verrucaria devensis	x	
Vishniacozyma sp.	x	
Vishniacozyma globispora	x	
Vishniacozyma victoriae	x	
Zasmidium queenslandicum	x	
Acrocalymma aquatica		x
Ascochyta maackiae		x
Aspergillaceae sp.		x
Botryosphaeriaceae sp.		x
Candida amphicis		x
Corynespora smithii		x
Cosmospora gigas		x
Daldinia starbaeckii		x
Dothistroma pini		x
Fusicolla sp.		x
Hannaella kunmingensis		x
Hypocreaceae sp.		x
Lecanicillium longisporum		x
Meira geulakonigii		x
Mycotribulus mirabilis		x
Pestalotiopsis rhododendri		x
Phacidium lacerum		x
Pseudopestalotiopsis theae		x
Pseudozyma hubeiensis		x
Pyrenochaetopsis leptospora		x
Trichoderma harzianum		x
Xylaria sp.		x
Xylariaceae sp.		x

S6.1 Pairwise PERMANOVA among the groups of the factor 'host plant orders'

PERMANOVA: Pairwise, factor 'host plant order'		
Pairs	**r^2**	**P***
Malpighiales vs Asterales	0.064	0.061
Malpighiales vs Dilleniales	0.073	**0.045**
Malpighiales vs Sapindales	0.064	0.106
Malpighiales vs Gentianales	0.076	**0.045**
Malpighiales vs Fabales	0.073	**0.045**
Malpighiales vs Ericales	0.188	**0.011**
Asterales vs Dilleniales	0.077	0.481
Asterales vs Sapindales	0.243	0.068
Asterales vs Gentianales	0.258	**0.045**
Asterales vs Fabales	0.133	0.216
Asterales vs Ericales	0.437	**0.011**
Dilleniales vs Sapindales	0.400	0.072
Dilleniales vs Gentianales	0.381	**0.049**
Dilleniales vs Fabales	0.251	0.182
Dilleniales vs Ericales	0.574	**0.021**
Sapindales vs Gentianales	0.406	0.221
Sapindales vs Fabales	0.645	0.350
Sapindales vs Ericales	0.562	0.068
Gentianales vs Fabales	0.525	0.221
Gentianales vs Ericales	0.526	**0.045**
Fabales vs Ericales	0.655	0.068
*Benjamini-Hochberg false discovery rate adjustments to protect pairwise PERMANOVA against false positives		

S6.2 Pairwise PERMANOVA among the combined factor 'host plant orders' and 'sap feeding insect' presence (SFI+) or absence (SFI-)

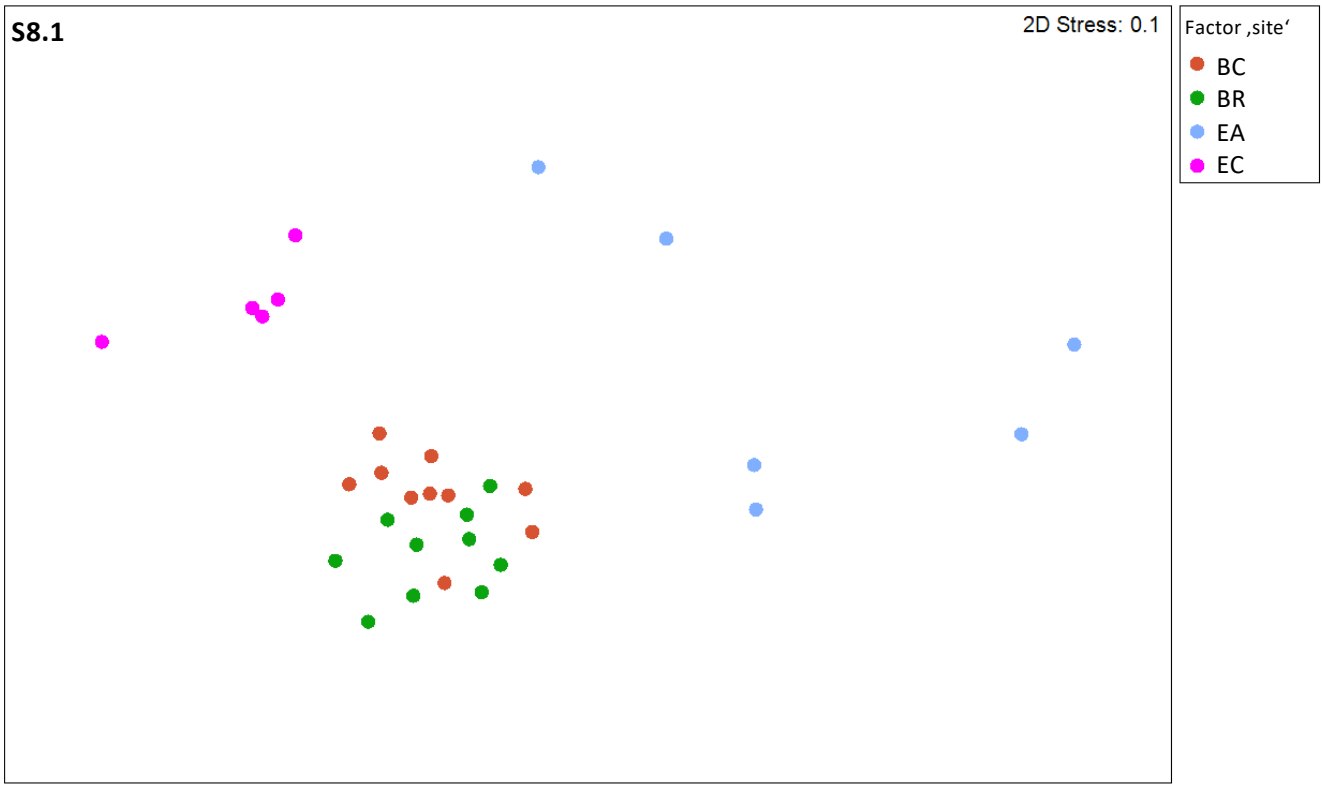
PERMANOVA: Pairwise, combined factor 'host plant order' and 'SFI' only pairs with significant results are shown		
Pairs	**r^2**	**P***
Malpighiales-SFI+ vs Malpighiales-SFI-	0.143	**0.009**
Malpighiales-SFI+ vs Ericales-SFI-	0.373	**0.014**
Asterales-SFI+ vs Malpighiales-SFI-	0.138	**0.009**
Asterales-SFI+ vs Ericales-SFI-	0.446	**0.024**
Dilleniales-SFI+ vs Malpighiales-SFI-	0.190	**0.009**
Dilleniales-SFI+ vs Ericales-SFI-	0.574	**0.031**
Malpighiales-SFI- vs Sapindales-SFI-	0.114	**0.036**
Malpighiales-SFI- vs Gentianales-SFI-	0.135	**0.036**
Malpighiales-SFI- vs Ericales-SFI-	0.269	**0.009**
*Benjamini-Hochberg false discovery rate adjustments to protect pairwise PERMANOVA against false positives		

S7 Pairwise PERMANOVA among the groups of the factor 'host plant family'

PERMANOVA: Pairwise, factor 'host plant family'		
Pairs	**r^2**	**P***
Chrysobalanaceae vs Asteraceae	0.122	0.179
Chrysobalanaceae vs Malpighiaceae	0.106	0.319
Chrysobalanaceae vs Dilleniaceae	0.195	0.117
Chrysobalanaceae vs Euphorbiaceae	0.230	0.190
Chrysobalanaceae vs Peraceae	0.179	0.199
Chrysobalanaceae vs Anacardiaceae	0.234	0.190
Chrysobalanaceae vs Apocynaceae	0.252	0.095
Chrysobalanaceae vs Fabaceae	0.241	0.272
Chrysobalanaceae vs Salicaceae	0.314	**0.050**
Chrysobalanaceae vs Ericaceae	0.406	**0.018**
Asteraceae vs Malpighiaceae	0.113	0.153
Asteraceae vs Dilleniaceae	0.077	0.501
Asteraceae vs Euphorbiaceae	0.248	0.078
Asteraceae vs Peraceae	0.192	0.095
Asteraceae vs Anacardiaceae	0.243	0.099
Asteraceae vs Apocynaceae	0.258	**0.034**
Asteraceae vs Fabaceae	0.133	0.229
Asteraceae vs Salicaceae	0.286	**0.033**
Asteraceae vs Ericaceae	0.437	**0.018**
Malpighiaceae vs Dilleniaceae	0.177	0.103
Malpighiaceae vs Euphorbiaceae	0.212	0.153
Malpighiaceae vs Peraceae	0.171	0.132
Malpighiaceae vs Anacardiaceae	0.233	0.117
Malpighiaceae vs Apocynaceae	0.241	0.078
Malpighiaceae vs Fabaceae	0.197	0.190
Malpighiaceae vs Salicaceae	0.306	**0.018**
Malpighiaceae vs Ericaceae	0.397	**0.034**
Dilleniaceae vs Euphorbiaceae	0.412	0.101
Dilleniaceae vs Peraceae	0.301	0.078
Dilleniaceae vs Anacardiaceae	0.400	0.095
Dilleniaceae vs Apocynaceae	0.381	0.059
Dilleniaceae vs Fabaceae	0.251	0.199
Dilleniaceae vs Salicaceae	0.366	0.050
Dilleniaceae vs Ericaceae	0.574	**0.034**
Euphorbiaceae vs Peraceae	0.298	0.153
Euphorbiaceae vs Anacardiaceae	0.485	0.340
Euphorbiaceae vs Apocynaceae	0.411	0.229
Euphorbiaceae vs Fabaceae	0.621	0.340
Euphorbiaceae vs Salicaceae	0.431	0.095
Euphorbiaceae vs Ericaceae	0.548	0.095
Peraceae vs Anacardiaceae	0.304	0.153
Peraceae vs Apocynaceae	0.277	0.324
Peraceae vs Fabaceae	0.402	0.153
Peraceae vs Salicaceae	0.310	0.078
Peraceae vs Ericaceae	0.429	**0.050**
Anacardiaceae vs Apocynaceae	0.406	0.229
Anacardiaceae vs Fabaceae	0.645	0.340
Anacardiaceae vs Salicaceae	0.404	0.100
Anacardiaceae vs Ericaceae	0.562	0.095
Apocynaceae vs Fabaceae	0.525	0.229
Apocynaceae vs Salicaceae	0.367	0.093
Apocynaceae vs Ericaceae	0.526	**0.043**
Fabaceae vs Salicaceae	0.541	0.100
Fabaceae vs Ericaceae	0.655	0.086
Salicaceae vs Ericaceae	0.590	**0.033**

*Benjamini-Hochberg false discovery rate adjustments to protect pairwise PERMANOVA against false positives

S8 Figues and tables of single species barcoding approach



S8.1: Non-metric multidimensional scaling (NMDS) ordination plot showing the compositional differences of the sooty mould communities from the different sampling sites North-eastern Brazil Caatinga (BC), North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA) and Central Europe colline zone (EC) (Jaccard similarities, stress = 0.1).

S8.2a

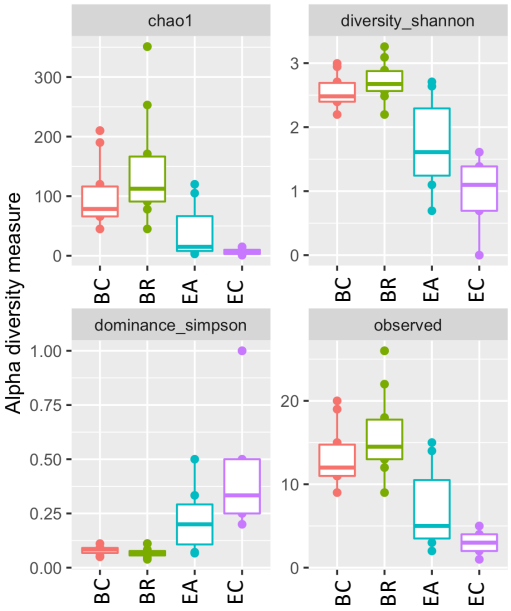
PERMANOVA: Global		
Factors	r^2	p
Site	0.356	0.002
Continent	0.163	0.001
SFI	0.090	0.012
Host plant family	0.370	0.01
Host plant order	0.259	0.003

S8.2b

PERMANOVA: Pairwise, factor 'site'		
Pairs	r^2	P*
BC vs BR	0.62	0.188
BC vs EA	0.296	0.003
BC vs EC	0.271	0.005
BR vs EA	0.303	0.003
BR vs EC	0.305	0.004
EC vs EA	0.263	0.038

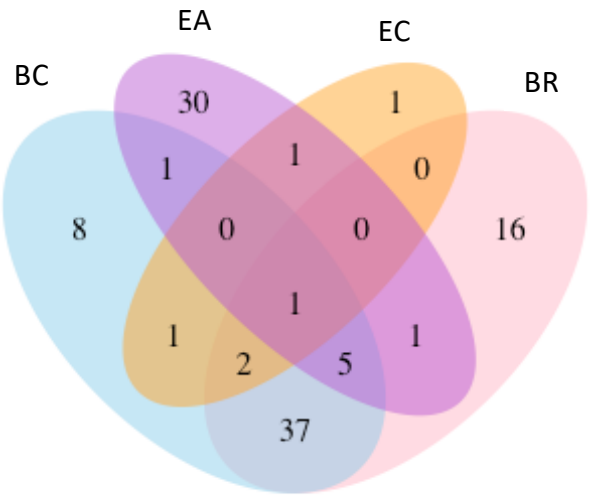
*Benjamini-Hochberg false discovery rate adjustments to protect pairwise PERMANOVA against false positives

S8.3a

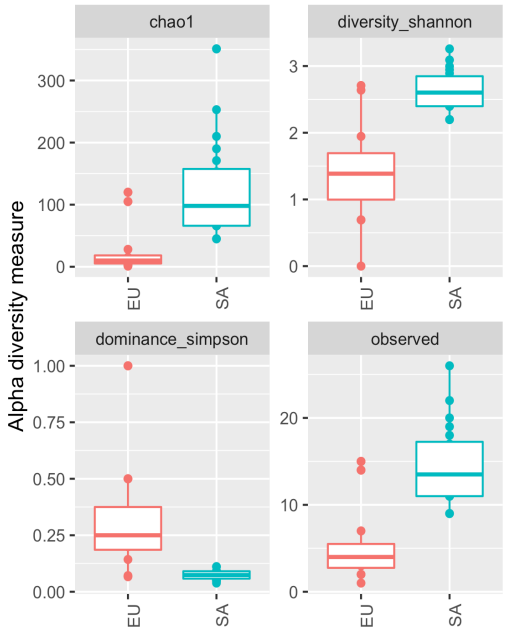


S8.3a-b: α-diversity, Venn diagramm based on factor ‚site': North-eastern Brazil Caatinga (BC) North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA) and Central Europe colline zone (EC)

S8.3b

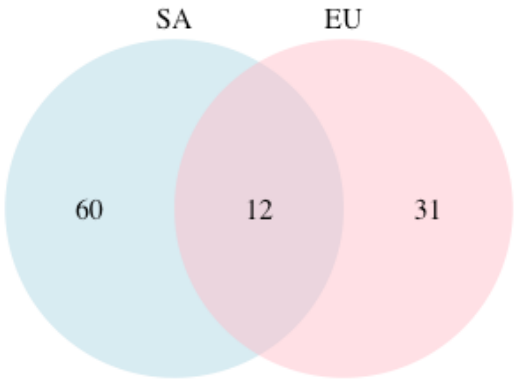


S8.4a

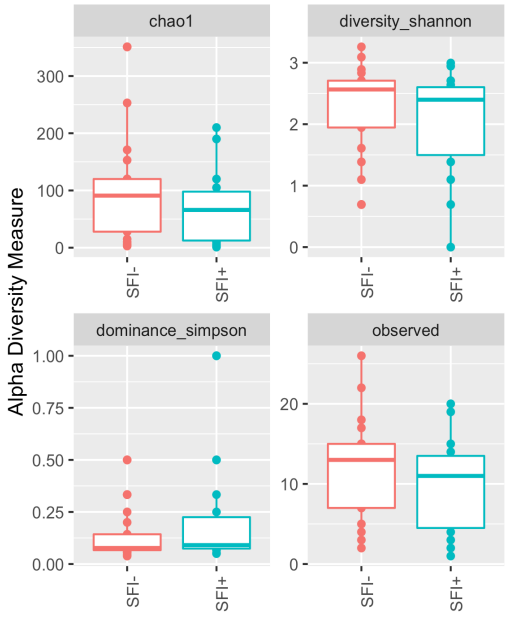


S8.4a-b: α-diversity, Venn diagramm based on factor ‚continent': Europa (EU) and South America (SA)

S8.4b

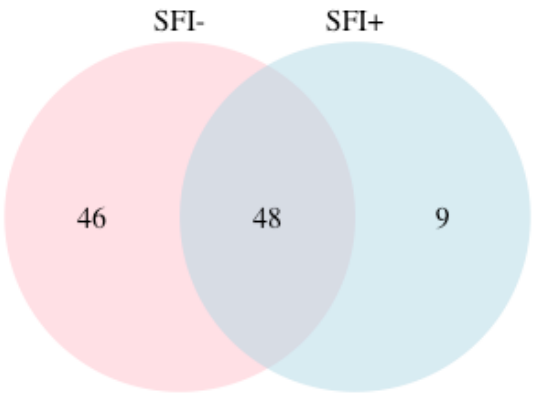


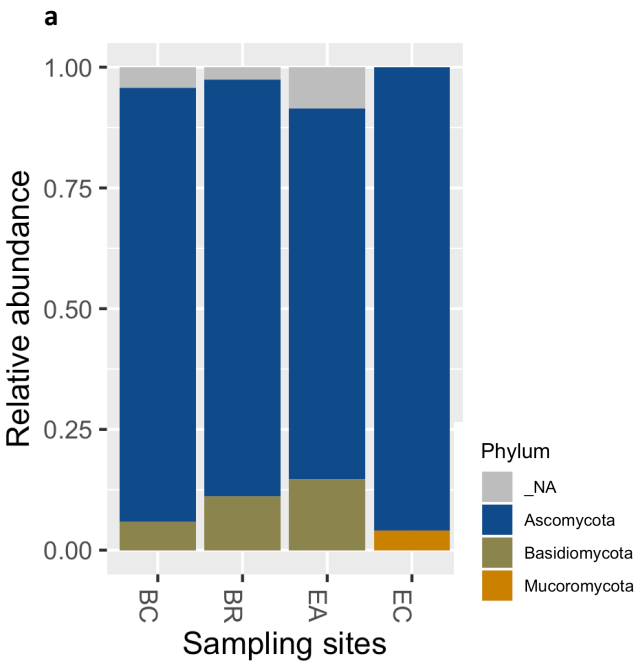
S8.5a

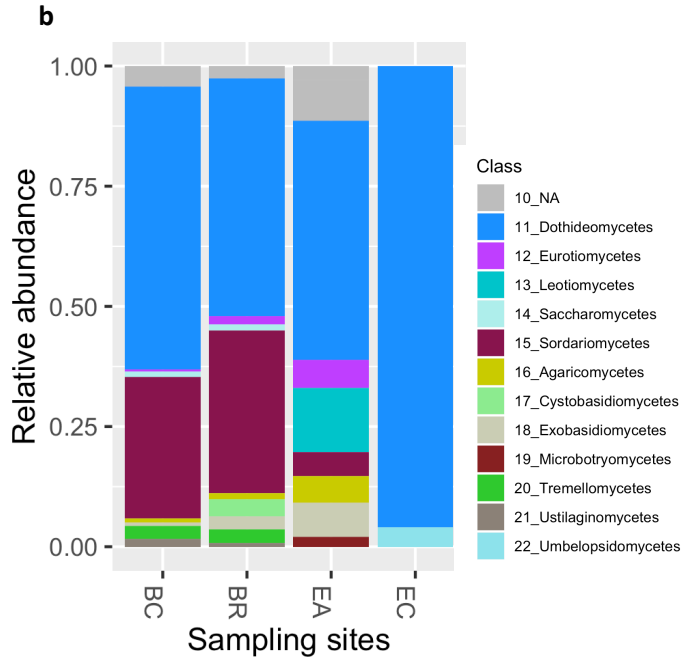


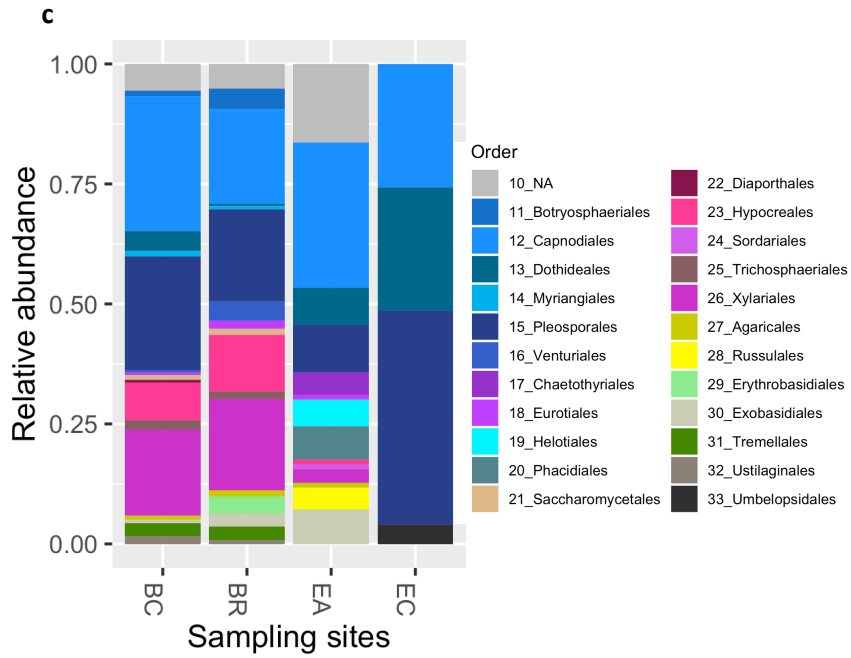
S8.5a-b: α-diversity, Venn diagramm plot based on factor sap-feeding insect presence (SFI+) or absence (SFI-)

S8.5b

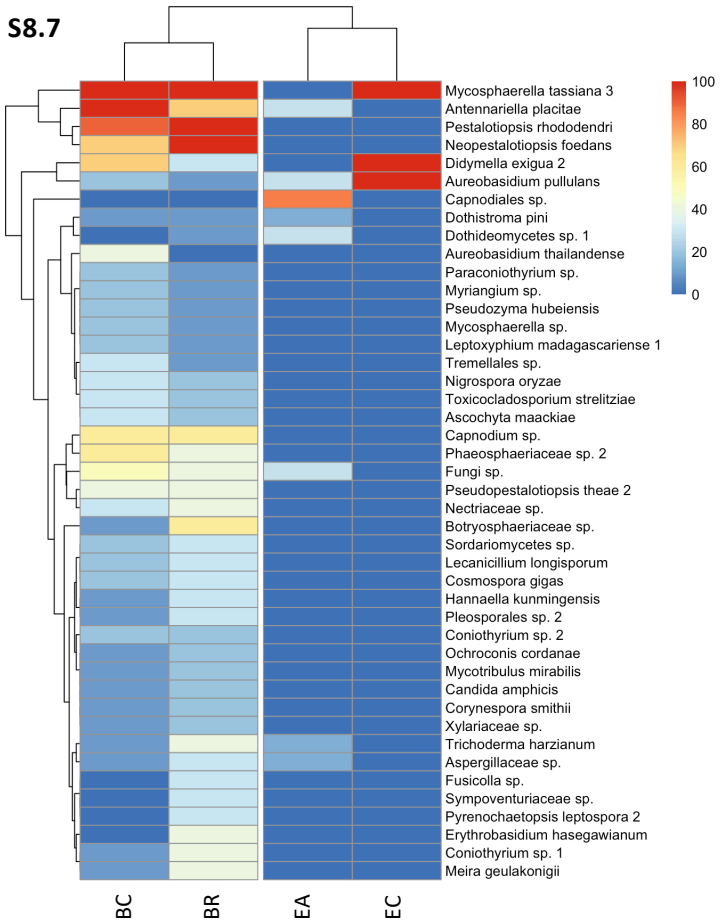




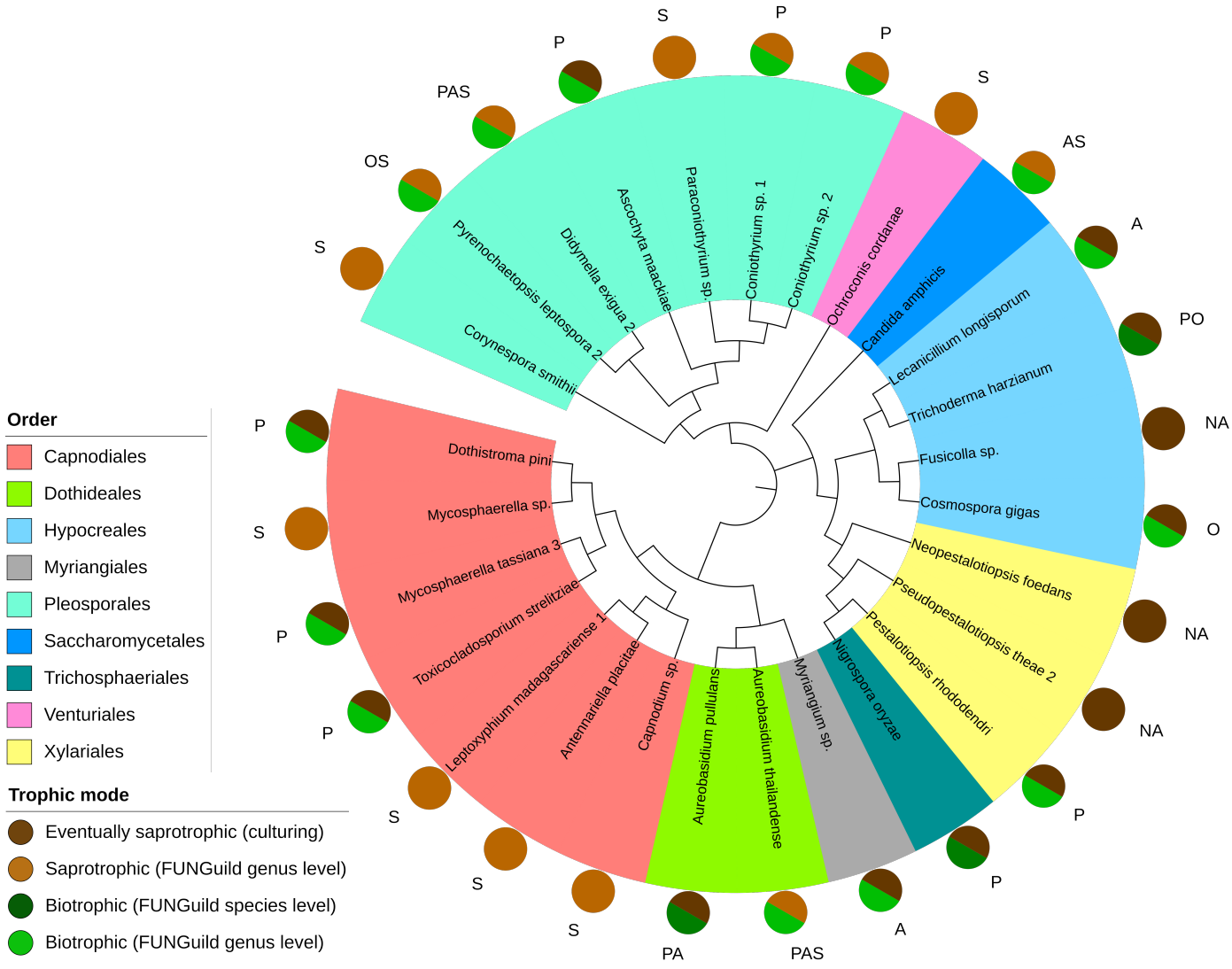




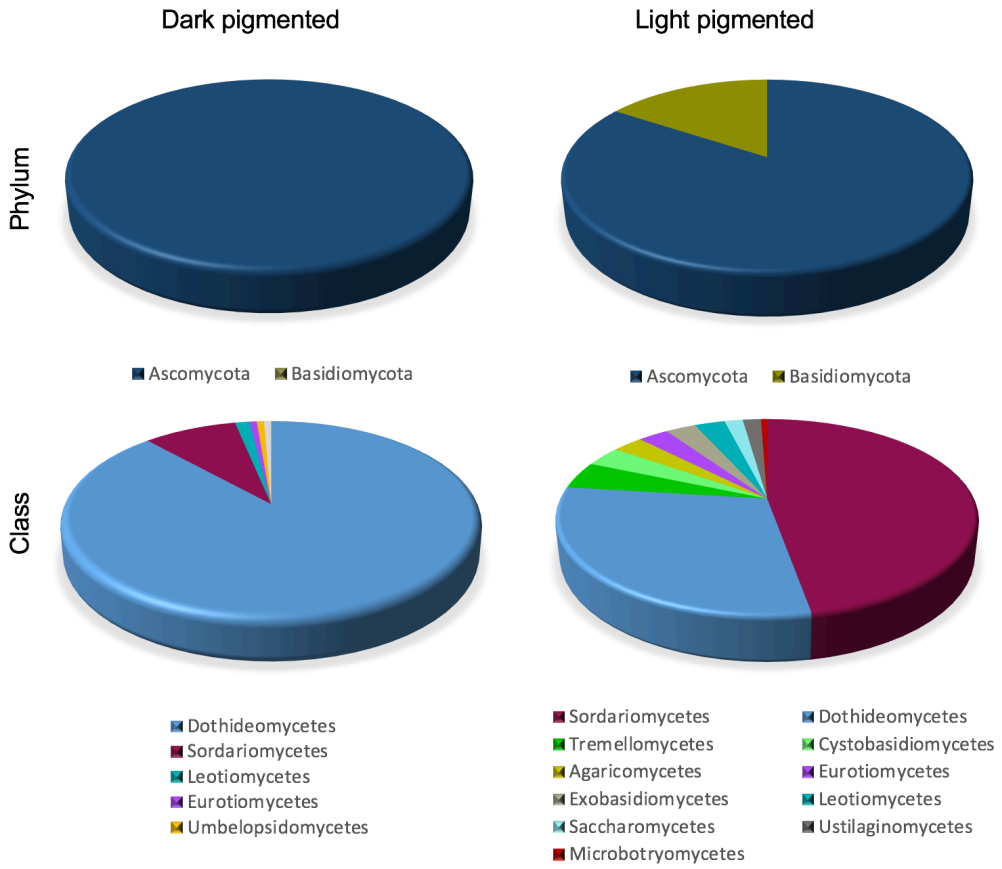
S8.6a-c: Barplots of the relative abundance of phyla (a), class (b) and order (c) in North-eastern Brazil Caatinga (BC) North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA) and Central Europe colline zone (EC) samples.



S8.7: Heat-map analysis of the 10% most common taxa based on their consistency (%) in North-eastern Brazil Caatinga (BC) North-eastern Brazil Tropical Rainforest (BR), Europe alpine zone (EA) and Central Europe colline zone (EC) samples



S8.8: Most abundant OTUs (occur in at least 3 samples) among the Ascomycota with taxonomic assignment on species or genus level and with FUNGuild classification on species, genus or family level. Tree was generated with maximum-likelihood method by Mega7 and visualized with iTOL (interactive Tree of Life). P: Plant-associated (including litter saprotrophic and wood saprotrophic), A: animal-associated, S: saprotrophic, O: 'other' (i.e. unspecific, fungus-associated, lichen-associated, dung saprotrophic), NA: no FUNGuild entry for this taxa available.



S8.9 Relative amount of species among phyla and class. Species from species barcoding are grouped in classes and phyla by pigmentation type and based on the relative amounts of samples they were found in.

12.4 Manuscript 4

Flessa F., Kehl A., W. Babel, G. Rambold, M. Kohl (2021): Effects of sap-feeding insects, plant characteristics and weather parameters on sooty moulds in the temperate zone. Manuscript in preparation for submission to Oecologia.

Effect of sap-feeding insects, plant characteristics, and weather parameters on sooty moulds in the temperate zone

Fabienne Flessa[1†], Wolfgang Babel[2], Alexandra Kehl[3], Gerhard Rambold[1], Matthias Kohl[4]

[1] University of Bayreuth, Dept. of Mycology, Universitätsstraße 30, D-95447 Bayreuth, Germany

[2]University of Bayreuth, Dept. of Micrometeorology, Universitätsstraße 30,
D-95447 Bayreuth, Germany

[3]Botanischer Garten der Universität Tübingen, Hartmeyerstr. 123
D-72076 Tübingen, Germany

[4]University of Furtwangen, Dept. of Medical and Life Science, Jakob-Kienzle-Str. 17
D-78054 Villingen-Schwenningen, Germany

[†]Corresponding author
Fabienne.Flessa@uni-bayreuth.de

Authors' contributions: FF originally formulated the idea, AK and FF developed the design of the field study, AK and FF conducted and monitored the fieldwork, MK performed statistical analysis, WB prepared the weather parameters (selection and calculation), FF wrote the manuscript under guidance of GR, considering the comments of all co-authors.

Abstract

In this study, relevant variables were determined to predict the probability of sooty mould biofilm (SMB) occurrence on willow leaves in a multivariate model. Despite a large temporal gap between the main sap-feeding insect (SFI) season in spring and the main season of SMBs in late summer and fall, trees infested with SFIs on four or more observation dates had significantly more SMBs (3.7-fold) toward the end of the growing season. In addition, a horizontal leaf position and recessed leaf veins, traits that may increase the wettability of the leaves, positively influenced the occurrence. Among the weather parameters, those that lead to higher moisture on leaf surfaces, namely high morning and evening relative humidity, higher precipitation sums, and lower vapour pressure deficit values, had the strongest positive effect. In addition to these parameters, higher wind speeds also had a strong positive effect on SMB occurrence.

Key words
Biofilm ecology, host plant influence, willow, pest management, climatic parameters

Introduction

Leaves and steams of higher plants are regularly colonized by sooty mould biofilms (SMBs), which are characterized by a superficial dark mycelium (Schoulties 1980; Callan and Carris 2004; Agrios 2005; Chomnunti et al. 2014; Kim 2016; Kim and Kim 2017). These fungi have a predominantly or temporarily saprotrophic life habit due to the absence of specific interbiotic cellular structures, such as haustorial protrusions, that are present in truly phytopathogenic fungi (An et al. 2006; Dekhuijzen and Scheer 1969), and they are not host plant specific (Callan and Carris 2004; Agrios 2005). Sooty moulds are not considered plant parasites; therefore, they do not penetrate the leaf surface (Hughes 1976; Agrios 2005; Chomnunti et al. 2014; Kim 2016; Kim and Kim 2017). Within phytopathology, they are considered a non-parasitic disease (Blancard 2012) due to its negative consequences for the host plant and economic damage, e.g., in tropical and subtropical crops, such as carambola, citrus, durian, durum wheat, guava, mango, and tomatoes (Swirski et al. 1997a, b; Ancisco et al. 2002; Siriphanich 2011; Warren and Sargent 2011; Blancard 2012; Fernandez and Knox 2012). They reduce respiration, and increase the water costs for each CO_2 molecule fixed during photosynthesis (Blancard 2012; Insausti and Ploschuk 2018). The black coating acts as a light filter, which leads to a reduction in incident light through physical obstruction (de Filho and Paiva 2006; Nelson 2008), resulting in an increase of leaf temperature (Wood et al. 1988) at least in the overgrown regions (Kim and Kim 2017) and a decrease of water content in covered leaves (Santos et al. 2013). The negative impact on photosynthesis of the affected plants (de Filho and Paiva 2006; Wood et al. 1988), however, is controversial (Insausti and Ploschuk 2018). At a

minimum, the plant needs to compensate for this disadvantage with changes in the morphology of cell organelles by more chlorophyll (Insausti et al. 2015; Kim and Kim 2017).

In general, it is postulated, that SMBs are strongly linked to the occurrence of members of the sap-feeding insect guild (SFI), which produce honeydew, a highly concentrated sugar dilution that may function as a nutritional resource for the fungal species (Hughes 1976; Parbery and Brown 1986; Callan and Carris 2004; Perez et al. 2009; Kirk et al. 2010; Dhami et al. 2013; Chomnunti et al. 2014). Therefore, it is recommended to reduce SMB accumulation indirectly by controlling SFIs with insecticides (Agrios 2005). The correlation between SFIs and SMBs has thus far only been studied for evergreen plants, foremost in the tropics and subtropics. Most studies examined variables that alter the composition of the fungal community, but not which variables lead to an occurrence on plants in the first place. Studies on multitrophic interactions in the phyllosphere, concerning SMB and SFIs, include usually just one (Stadler et al. 1998; Mühlberg and Stadler 2005; Perez et al. 2009; Shukla et al. 2017) or a few host plant species (Dhami et al. 2013) and do not provide conclusions about SMB-promoting host plant characteristics. The occurrence, structure, and succession of epifoliar fungal communities are known to underlie seasonal variations (Cabral 1985; Lee and Hyde 2002; Osono 2008), but data on SMBs were usually sampled during a very short time period and, therefore, disregard seasonal variations in SFI dynamics (Perez et al. 2009; Dhami et al. 2013). In some studies on symptomless leaves without visible SMBs, variations in fungal communities were investigated and mainly interpreted as directly linked with climatic weather conditions, such as temperature, precipitation, relative humidity, and wind speed (Lee and Hyde 2002; Talley et al. 2002; Gomes et al. 2018) without a link to SFI occurrence, but there was one long-term study in mango orchards that confirmed a strong correlation between SFIs and SMBs (Shukla et al. 2017) in combination with weather parameters. However, since this study was carried out on a single evergreen host plant species in the tropics, no conclusions can be drawn about the influence of plant characteristics, especially on deciduous leaves in the temperate zone. While host plants in tropical habitats are usually perennial and have durable leaves, they also provide a continuous habitat for sooty moulds. This continuity does not exist in deciduous plant leaves. The development of the fungal community stops in autumn and restarts every spring with the leaves sprouting (Flessa et al. 2012). As SMBs are a more subtropical or tropical (Callan and Carris 2004; Kirk et al. 2010) than a temperate climatic zone phenomenon, the present study is the first on deciduous host plants that includes a broad range of species of one genus of host plants, i.e., *Salix* (willows), and examines the effects of SFI occurrence on their development in combination with leaf traits and weather parameters. Willows are widely distributed in the northern hemisphere, mainly in the temperate climatic zone, i.e., in the northern parts of Europe and North America, as well as Asia, with the diversity center located in China. Willows are uncommon in subtropical and tropical regions, and only a few species have been reported in Africa and South America (Argus 1986; Zhen-Fu 1987; Ghahremaninejad et al. 2012; Zhao et al. 2019). They are suitable as study objects, as they are known potential host plants of SMBs (Hughes 1976; Flessa et al. 2012; Khodaparast et al. 2015) and aphids (Blackman and Eastop 2000) and differ considerably in

their interspecific leaf characteristics. The main saprotrophic fungi within the SMB on leaves of willows in the studied area are *Aureobasidium pullulans*, *Cladosporium cladosporioides,* and *Phoma* sp., with *A. pullulans* being the most dominant species (Flessa et al. 2012).

The aim of our study was to identify, as a first step, those variables that have a significant impact on the development of SMBs. In a second step, our goal was to develop a multivariate model based on the relevant variables that would allow predictions of their occurrence on the leaves of willows.

Material and Methods

Location of the hostplants:

Data sampling took place during one vegetation season from May to September 2010 in the *Salix* collection (Salicetum) of the Bayreuth University Ecological-Botanical Garden (49°55'31.5" N, 11°34'58.6" E, 352 m alt.). The area of the study site measured 4010 m^2. All plants were grown without manipulation, i.e., application of pesticides or fertilizers or truncation.

Observation in the field and data collection:

This study included 147 host plants, belonging to 75 different taxa and hybrids within the plant genus *Salix,* which were all exposed to the same conditions (geographical location, treatment).

Data sampling was performed at bi-weekly intervals from the calendar weeks CW 18 until CW 36. On each date, for each plant, the presence of SMB (yes/no), SFIs (yes/no; sporadic, at least 10 individuals, heavy infestation of the whole plant, i.e., about 50% of leaves/shoots are infested), and evidence of ants visiting SFIs by (yes/no) were registered.

The following characteristics of host plant leaves were recorded in August: summer leaf position (upwards, horizontally, downwards), summer leaf structure (smooth or rugose), leaf appearance (glabrous leaf blade, light-haired, dense-haired, glaucous), adaxial leaf vein type (flat, concave, convex), adaxial midrib type (flat, U-shaped; (Mantovani et al. 2009), adaxial midrib pubescence (yes, no), and visible adaxial surface gloss (yes, no).

The plant characteristics from surveys were supplemented with further data, i.e., leaf length and form, leaf glands (yes/no and size small, medium, large), surface waxes (yes/no), shape of the leaf (narrow, narrow-broad, broad, elongated, round), from the literature (Rechinger 1962; Stelfox 1965; Chmelar and Meusel 1976; Walters 1984; Lautenschlager-Fleury and Lautenschlager-Fleury 1988; Lautenschlager 1989; Uchytil 1992; Böhlmann 2012; Isebrands and Richardson 2014; Roloff and Bärtels 2018; Mastel 2019; Züllig-Morf 2019) and online resources (arbolapp.es, lhprism.org, rhs.org, gobotany.nativeplanttrust.org, treesandshrubsonline.org, eFloras.org).

Climatic data:

Climate data were provided by the Micrometeorology group at the University of Bayreuth and derived

from a weather station in the Ecological-Botanical Garden of the University of Bayreuth, at a distance of about 250 m to the middle of the *Salix* collection area (49°55'45" N., 11°35'10" E., 350 m. alt.). The data we used to analyze the impact of different climatic factors on SMBs were
daily precipitation sum (mm), mean air temperature (°C), daily air temperature minimum and maximum (°C), global radiation (MJ-m2), sunshine duration in hours, mean vapor pressure (hPa), daily relative humidity minimum (%), instantaneous 10 min average of relative humidity at 8, 12, 16, and 20 MEZ (%), daily mean wind speed (m-s), vapor pressure deficit (hPa), daily sum of potential evaporation rates (Ep, Priestley-Taylor, mm) and actual evaporation rate (ET, Penman-Monteith, mm). For each measurement, we calculated mean values or totals over the 14, 7, and 3 days preceding the collection date.
The measurements were obtained as follows: air temperature (2 m height): Frankenberger psychrometer, actively ventilated; precipitation (1 m height): OTT pluvio, global radiation: Kipp&Zonen CM14. Air pressure (1 m height): Ammonit AB60; vapor pressure and relative humidity: Vaisala HMP, corrected with summertime psychrometer measurements; direct radiation fraction and sunshine duration (2 m height): Thies SDE 9.1; wind velocity and direction (17 m height): Thies 2d Ultrasonic. Potential evaporation rates were calculated after Priestley-Taylor.

Statistical analyses:

Analyses were conducted using the packages 'lme4' (Bates et al. 2015), 'ggeffects' (Lüdecke 2018), and 'oddsratio' (Schratz 2017) of the statistical software R v. 4.0.3 (R Core Team 2020). Logistic mixed effect models were used for the analysis. The influence of SFI observations, ant presence, and different plant characteristics on the occurrence of SMB were initially evaluated univariately. For the multivariate model, in a first step, variables that were significant ($p < 0.05$) or almost significant ($p < 0.1$) were selected. We had to restrict our considerations to multivariate logistic mixed-effects models with few variables (4-5, depending on the variables), since the fitting algorithm became instable leading to convergence problems for higher numbers of variables. Univariate testing was also initially performed on the weather variables. Due to the limited number of variables, only one weather variable could be included in the multivariate model at a time. The selection was based on biological relevance in comparable studies, significance of the corresponding p-value, and a low AIC value.

Results

The main occurrence of SMBs, as well as the main occurrence of SFIs on willows, was highly seasonal. The SFI main season started with a peak in mid-May and lasted until mid-June. The curve for the SMBs showed a shifted pattern with an increase from mid-August to September, when the insects were rarely found (Fig. 1). There was a large time gap between both main seasons in June and the sudden increase of biofilm records in August.

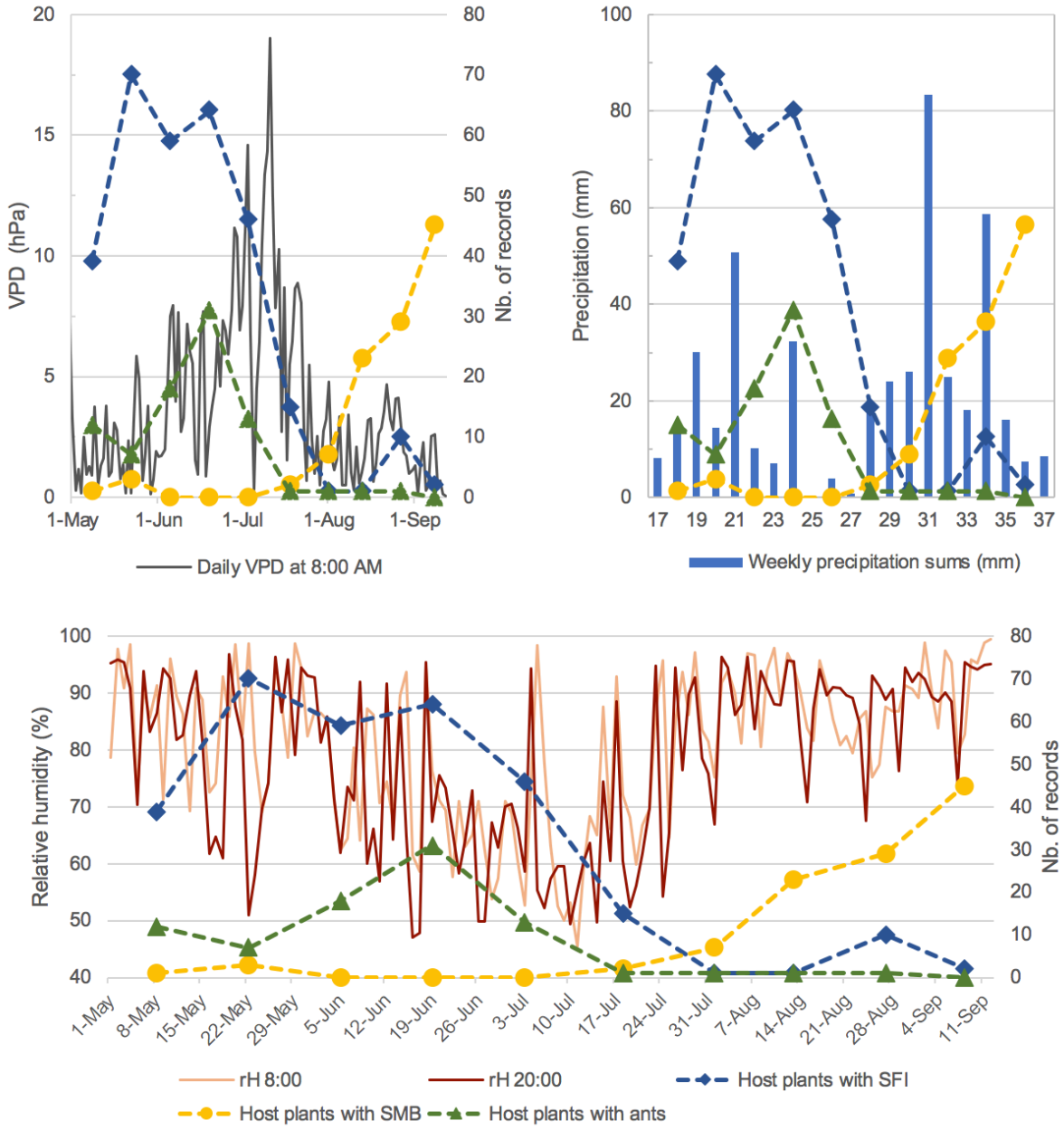


Figure 12 Seasonality of appearance of sooty mould biofilms (SMB), sap-feeding insects (SFI), and ants during the sampling period in relation to climatic data. A) Weekly sum of precipitation, B) daily means of maxima and minima of air temperature, C) daily means of relative morning and evening humidity.

On 35% of the 147 willow individuals in this study, SMB was observed at least at one collection date, and 86% were infested with SFI at one or multiple observation dates. Among the willow species, subspecies, and hybrids that were represented by at least 3 individuals in the experimental design, SMB was observed on more than half of the individuals, i.e., *S. helvetica* Vill., *S. appendiculata* Vill., *S.* x

hegetschweileri Heer, *S. caprea* L., and *S. cinerea* L., but on one-third, it was never observed, i.e., *S. apennina* Skvortsov, *S. caesia* Vill., *S. glabra* Scop., *S. mielichhoferi* Saut, and *S. repens* L.

SMBs recorded without previous or simultaneous SFIs were observed in only 2% of host plants. However, the occurrence of SFIs did not necessarily result in SMBs later in the season; half of the plants on which SFIs were detected never showed SMBs during the study period. While the occasional occurrence of SFIs had no significant effect, their presence on four or more observation dates significantly increased the probability of an occurrence ($\beta = 1.515$, $z = 2.86$, $p = 0.004$, AIC = 715.3) (ESM 2). The probability of developing an SMB was 3.7-fold higher on trees with ≥ 4 SFIs than on trees with less observations. Of the hosts listed above, with at least three individuals without SMBs, only a single individual of *S. glabra* was observed with SFIs four times, all others belonged to the willows with less than four observations.

Whether SFIs were visited by ants or not had no significant impact on SMBs. Of the plant characteristics examined, a horizontal leaf position ($\beta = 1.968$, $z = 2.56$, $p = 0.011$, AIC = 704.8) and rough leaves ($\beta = 0.842$, $z = 2.05$, $p = 0.04$, AIC = 711.1) had a positive significant effect; sunken leaf veins had a positive effect, but it was only almost significant ($\beta = 0.903$, $z = 1.89$, $p = 0.058$, AIC = 665). Longer leaves also had a positive effect, which increased with leaf length, but was also only almost significant (category with the longest leaves of 10–15 cm: $\beta = 1.257$, $z = 1.93$, $p = 0.054$, AIC = 676.2). While most leaf shapes had no significant effect, wide leaves had a negative effect ($\beta = -1.528$, $z = -2.3$, $p = 0.021$, AIC = 653.1). All other plant characteristics had no significant effect (ESM 2). With the significant and almost significant variables, we first built a multivariate model. We then successively removed the variable with the lowest p-value from the model, resulting in a model with SFI observations on four or more dates, horizontal leaf position, and concave leaf veins (Table 1). The relative abundance of SMBs on trees with an SFI ≥ 4, horizontal leaf position, and recessed veins was 30%, and the relative abundance predicted by the model was 35% (CI 15–63%). In comparison, the observed relative frequency of SMBs on trees with an SFI <4, without horizontal leaf position and recessed leaf veins was 2.5%; the relative frequency predicted by the model was 1.2% (CI 0.5–3.1%).

In the next step, we added weather variables to this model. Due to convergence problems with the fitting algorithm, we could only add one variable at a time. Therefore, we built different models by adding the weather variables individually (ESM 3). Weather parameters that, in combination with the plant model (SFI ≥ 4, horizontal leaf position, and recessed veins), led to the highest (>80%) predicted relative abundances of the SMBs were mean VPD of 3 hPa at 8:00 am over the last three days (90%, CI 65–98%), increased wind speed of 2.1 m-s over the last three days (89%, CI 64–97%), high relative humidity at 8:00 AM of 90% over the last 14 days (87%, CI 56-97%), high precipitation sum over the last 14 days of 85–90 mm (89%, CI 52–97%), and high relative humidity at 8:00 PM of 91% over the last 7 days (82%, CI 48–95%).

Favorable weather conditions only led to an increased probability of the occurrence when they occurred in combination with prolonged or repeated SFI presence and favorable plant characteristics.

Based on the multivariate model, the predicted probability of SMB occurrence at a morning relative humidity of 90% over the past 14 days was 29-fold higher for trees with ≥ 4 SFI observations, horizontal leaf position, and recessed leaf veins than for trees with < 4 SFI observations, a non-horizontal leaf position, and non-recessed leaf veins. This occurrence was still 9.9-fold higher for trees with favorable plant traits but without SFI ≥ 4 (Table 2).

Among the other weather variables that were univariately significant, with lower predicted probabilities on SMB occurrence by the multivariate model compared to the parameters mentioned above, lower average values of ETp (2.1 mm), lower average values of GLB (12 MJ-m2), a medium to shorter mean sunshine duration (3.8 to 6 h), and medium averages of the maximum daily temperature (21°C) appeared beneficial (data not shown).

Discussion

Applied methods:

Due to the study design, in which it was important for us to compare plants under similar conditions, the number of plant individuals was limited. We examined all plant individuals available on the plot and found that on two-thirds, no sooty mould occurred. Nevertheless, we could identify variables that lead to a higher probability for the occurrence of SMB. In addition, it was possible to derive a multivariate model in which all plant-related variables relevant for SMB were included. The observed relative abundance corresponds quite well with the relative abundance predicted by the model.

Unfortunately, it was not possible to include more than one weather parameter in this model because of convergence problems with the fitting algorithm.

Influence of SFI occurrence and host plant traits

In the tropics, SMB occurs throughout the year on evergreen host plants (Shukla et al. 2017), while in the temperate location studied, it can only be observed on deciduous leaves of native plants toward the end of the growing season. However, many weather parameters are similar in spring and fall and, therefore, cannot be the main reason for the late occurrence. They were found on slightly more than one-third of the experimental plants, and most individuals remained infestation-free during the observation season in our study. Since they can occur on almost all surfaces as soon as conditions are favorable and, in addition to honeydew, leachates from leaves can also serve as a nutritional source, it is possible that individual plant species have further traits that may prevent SMBs, in addition to the variables examined in this study. One possible reason may be salicylates, common secondary metabolites of willows, which at high doses (2.0–5.0 mM) can have a negative effect on the growth of individual fungal species (Strobel and Porter 2005). That effect of salicylates was confirmed in an in vivo experiment with *Aspergillus* (Panahirad et al. 2014) in *Pistacia vera* fruits. Conversely, *Epichloë festucae*, which is a symbiotic fungal endophyte of *Festuca rubra*, expresses a salicylate hydroxylase and is, therefore, able to bypass that host plant mechanism (Ambrose et al. 2015). Whether fungi of the core community of SMB

in the experimental area, i.e., *Aureobasidium pullulans* and *Cladosporium cladosporioides* (Flessa et al. 2012), are affected by salicylates needs to be examined in future studies. Since SMBs in this study were found on *S. pentandra*, and *S. purpurea*, which are willows with verified medium to high salicylate content (Förster et al. 2008; Volf et al. 2015), it can be presumed that salicylates cannot prevent their occurrence. Salicylate content in willows is highest at the beginning of the vegetative season in March and continues to decrease throughout the season (Förster et al. 2008), representing an inverse pattern with SMB occurrence, which could be a reason for the appearance of SMB towards the end of the season. No SMBs were found on *S. daphnoides,* which could be related to the significantly higher salicylate content compared to the other two willows. However, this remains speculative, as the salicylate content was determined in the bark and not in leaves by Förster et al. (2008), and only two individuals of *S. daphnoides* were present in our experimental design.

Another reason for the late occurrence of SMBs could be related to changes in surface structures during leaf aging. One of the most important ageing-related factors is the roughness of surfaces, which is much higher in older leaves (Mechaber et al. 1996). The process of getting rougher alters the surface topography, which has impact on key factors for epifoliar communities. It affects the surface wettability, temperature, humidity, and windspeed and can affect the localization of plant compounds released from cells on the leaf surface (Ford and Salt 1987; Derridj et al. 1989). If the wettability of a leaf changes during the aging process, this affects the fungal growth because it facilitates the germination and growth of germ tubes (Dickinson 1981, 1986; Cabral 1985). Horizontal leaf position, rugose leaf surfaces, and concave veins are plant traits that had significant or almost significant effects on SMB in univariate models. Those traits may lead to higher water availability and more available nutrients on leaves through the accumulation of honeydew or leachates.

While sooty mould fungi may also be associated with extrafloral nectaries (Ji-Hyun et al. 2015; Choi et al. 2015) or leaf glands (Flessa and Rambold 2013), we did not detect a significant effect of leaf glands on SMBs. This is in agreement with the results on *Juglans regia*, where SMBs were associated with honeydew of aphids but not with the presence of glandular trichomes (Kim 2016).

On mango in the tropics, SFI incidence significantly influenced SMB incidence up to 45% (Shukla et al. 2017), and SFIs often occurred simultaneously with the SMB (Dhami et al. 2013; Shukla et al. 2017), which could not be confirmed in this current study for the temperate region on host plants with deciduous leaves. However, despite the large time gap between both main seasons, trees infested by SFIs on four or more observation dates had significantly more SMBs (3.7-fold). On evergreen conifers in the temperate region, there were significantly more filamentous fungi found in the time when the aphids were most abundant as well (Stadler et al. 1998). In the month before (May), when aphid abundance was low, the abundance of microorganisms was low as well, but fungi were still more abundant on previous aphid-invested twigs than on the control twigs without aphids in September, which is consistent with the observations in our study. This influence partially be due to the honeydew produced by SFIs. Many SFIs on willows are phloem-feeders and belong to the aphids (Charles et al. 2014). Many

of them are known to decrease plant health (Dixon 1998), particularly in temperate regions (Blackman and Eastop 2000). Aphids secrete saliva when they pierce and feed on host plant tissue. This saliva contains effectors that are secreted into the host and manipulate cell processes in plant tissue. Aphid damage in plants can result in water stress, reduced growth, and wilting (Jaouannet et al. 2014) and could be an explanation for the increased SMB occurrence of these trees. One reason that less frequent SFI feedings (<4 observation dates) did not have a significant effect on SMB occurrence could be that honeydew produced by insects is, to a major proportion, consumed by other insects (Stadler and Müller 1996). About one-quarter to one-third of all aphids are myrmecophiles (Bristow 1991; Stadler 1997), and ants alone may consume more than two-thirds of the available honeydew (Müller 1956, 1960). However, no significant negative effect of ant visitation to SFIs could be detected on SMB occurrence in this study. This might need to be investigated in more detail in a future study, as a shorter observation interval is most likely required for adequate monitoring of ant presence.

Influence of weather

In the multivariate plant model combined with individual weather parameters, the highest probabilities of SMB occurrence were predicted at low VPD, higher but not maximal precipitation sums, and the highest values of morning and evening relative humidity. In tropical SMBs on mango, a positive relationship was found only for precipitation, while morning and evening relative humidity had no effect (Shukla et al. 2017). Increased precipitation also led to significantly higher infestations of leaves by biotrophic fungi that cause grapevine leaf disease (Serra et al. 2018). However, a positive effect of morning and evening relative humidity is known for the biotrophic fungus *Mycosphaerella berkeleyi,* which causes late leaf spot disease in groundnuts (Pappachan et al. 2015). In a study of cultivable fungi growing asymptomatically on leaf surfaces from a temperate region, low VPD and a daily relative humidity of >90% and higher monthly precipitation averages also had a positive effect on fungal abundance (Talley et al. 2002). These values all relate to water availability for fungi, which is an important factor for fungal growth (Ruinen 1961). This also applies to SMB as seasonal variations in *C. cladosporioides*, a fungal species belonging to the core community of SMBs on willows (Flessa et al. 2012), which are directly positively linked to relative humidity (Cabral 1985). Growth and survival of fungal spores germinating on leaves depends not only on the amount of moisture available but also on the length of time the leaf is wet. Although the duration of leaf wetness was not measured, the positive effects of horizontal leaf position and incised leaf veins on SMBs indicate that, in addition to higher nutritional availability produced by SFIs, available moisture plays a major role in the growth of sooty moulds on leaves. Rainfall and night dew form thicker water films on leaf surfaces (Burkhardt and Hunsche 2013), which enables chemical reactions between compounds dissolved in the rain or dew water and leachates from the leaf, impacting phyllosphere microorganisms by altering the water pH and availability of nutrients (Morris 2002). Honeydew becomes desiccated at a relative humidity of < 95% (Lievens et al. 2015). As honeydew is water soluble, rainfall washes it to underlying plant parts as a nutrition source

for SMB fungi (Beggs et al. 2005), where it will persist for long periods during dry periods (Batista and Ciferri 1963a). High prediction probabilities for SMBs also resulted from higher mean wind speeds 3 days before the observation date, in combination with the multivariate plant model. Although wind causes desiccation and, thus, a negative effect was expected, fungi belonging to the SMB core community, i.e., *A. pullulans*, *C. cladosporioides*, and *Phoma* sp., were regularly found in the spectrum of airborne fungal spores (Ogulana 1974; Górny et al. 2002; Horner et al. 2004). The influence of higher wind speed on the abundance and diversity of epiphytic microorganisms is consistent with other studies (Vacher et al. 2016; Gomes et al. 2018).

Conclusion

Even if SFIs and SMBs do not occur at the same time, SFIs have a significant impact on SMBs. Intervention of SFI infestation to prevent SMBs should be performed as early as possible, since a prolonged SFI presence will lead to increased SMB prevalence weeks after the SFIs have disappeared. However, the approach of simply reducing SFIs to reduce SMBs may fall short. In a greenhouse, good moisture management that prevents sooty mould growth is essential. In addition, when possible, care should be taken in breeding to obtain plants with leaf characteristics that are less conducive for SMBs.

Declarations

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Consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: All relevant data generated or analyzed during this study are included in this published article and its Electronic Supplemental Material (ESM 1).

Authors' contributions: FF originally formulated the idea, AK and FF developed the design of the field study, AK and FF conducted and monitored the fieldwork, MK performed statistical analysis, WB

prepared the weather parameters (selection and calculation), FF wrote the manuscript considering the comments of all co-authors.

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Tables

Table 1: Multivariate model with the relevant tree-based variables. A) Effects, B) predicted probabilities. SFI ≥ 4: trees with sap-feeding insect observations at 4 or more observation dates, LP: horizontal leaf position, LV: recessed leaf veins.

A)	Estimate	SE	Z	p-value		CI 95%
Intercept	-4.342	0.459	-9.47	<0.0001	***	-5.24, -3.44
SFI≥4	1.848	0.522	3.54	0.0004	***	0.83, 2.87
Horizontal leaf position	1.151	0.408	2.82	0.0048	**	0.35, 1.95
Concave leaf veins	0.748	0.42	1.78	0.0751	.	-0.08, 1.57

B)	Predicted	2.5%	97.5%
SFI≥4 = no, horizontal LP = no, concave LV = no	0.01	0.01	0.03
SFI≥4 = no, horizontal LP = no, concave LV = yes	0.08	0.03	0.20
SFI≥4 = no, horizontal LP = yes, concave LV = no	0.04	0.02	0.07
SFI≥4 = no, horizontal LP = yes, concave LV = yes	0.21	0.09	0.41
SFI≥4 = yes, horizontal LP = no, concave LV = no	0.03	0.01	0.07
SFI≥4 = yes, horizontal LP = no, concave LV = yes	0.15	0.05	0.36
SFI≥4 = yes, horizontal LP = yes, concave LV = no	0.08	0.04	0.16
SFI≥4 = yes, horizontal LP = yes, concave LV = yes	0.36	0.15	0.63

Table 2: Multivariate model with the relevant tree-based variables and relative morning humidity. A) Effects, B) predicted probabilities. SFI ≥ 4: trees with sap-feeding insect observations at 4 or more observation dates, LP: horizontal leaf position, LV: recessed leaf veins.

A)	Estimate	SE	Z	p-value		CI
Intercept	-6.3907	0.7307	-8.746	<0.0001	***	-7.82, -4.96
SFI≥4	2.6298	0.7563	3.477	0.000507	***	1.15, 4.11
Horizontal leaf position	1.5259	0.5473	2.788	0.005298	**	0.45, 2.60
Concave leaf veins	1.1269	0.5828	1.934	0.053154	.	-0.02, 2.27
poly(rH_8h_mean14d_perc, 2)1	74.923	15.9575	4.695	2.66E-06	***	43.65, 106.20
poly(rH_8h_mean14d_perc, 2)2	12.5631	9.4049	1.336	0.181614		-5.87, 31.00

B)	rH 64%	CI 2.5%	CI 97.5%	rH 70%	CI 2.5%	CI 97.5%	rH 77%	CI 2.5%	CI 97.5%	rH 83%	CI 2.5%	CI 97.5%	rH 90%	CI 2.5%	CI 97.5%
SFI≥4 = no, horizontal LP = no, concave LV = no	<0.001	<0.001	0.001	<0.001	<0.001	0.001	0.001	<0.001	0.003	0.003	0.001	0.012	0.033	0.010	0.103
SFI≥4 = no, horizontal LP = no, concave LV = yes	<0.001	<0.001	0.004	<0.001	<0.001	0.003	0.002	<0.001	0.008	0.010	0.002	0.037	0.096	0.029	0.274
SFI≥4 = no, horizontal LP = yes, concave LV = no	<0.001	<0.001	0.005	0.001	<0.001	0.004	0.003	0.001	0.009	0.014	0.005	0.038	0.137	0.063	0.273
SFI≥4 = no, horizontal LP = yes, concave LV = yes	0.001	<0.001	0.016	0.002	<0.001	0.012	0.008	0.002	0.030	0.043	0.014	0.122	0.328	0.141	0.593
SFI≥4 = yes, horizontal LP = no, concave LV = no	0.001	<0.001	0.019	0.002	<0.001	0.016	0.008	0.002	0.043	0.042	0.009	0.177	0.323	0.091	0.696
SFI≥4 = yes, horizontal LP = no, concave LV = yes	0.003	<0.001	0.059	0.006	0.001	0.048	0.025	0.005	0.127	0.118	0.025	0.415	0.596	0.214	0.889
SFI≥4 = yes, horizontal LP = yes, concave LV = no	0.004	<0.001	0.077	0.009	0.001	0.060	0.037	0.008	0.148	0.166	0.045	0.457	0.687	0.338	0.904
SFI≥4 = yes, horizontal LP = yes, concave LV = yes	0.012	0.001	0.218	0.027	0.004	0.179	0.106	0.022	0.382	0.381	0.110	0.754	0.872	0.557	0.973

Electronic Supplemental Material ESM

ESM 1: Complete dataset of all 10 observations in this study: sooty mould biofilm (SMB), sap feeding insect (SFI), and ant observations, plant characteristics, and weather variables as described in the Material and Methods.

ESM 2: Effects and predicted probabilities of sap feeding insects (SFI), ants, and plant characteristics on the occurrence of sooty mould biofilm (SMB) (univariate models).

ESM 3: Effects and predicted probabilities of multivariate models with one weather variable each, i.e., mean VPD over the last three days, mean wind speed over the last three days, precipitation sums over the last 14 days, relative morning humidity over the last 14 days, relative evening humidity over the last 7 days.

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ORIGINAL ARTICLE

Towards an efficient phenotypic classification of fungal cultures from environmental samples using digital imagery

Andrea Pietrowski · Fabienne Flessa · Gerhard Rambold

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Abstract The use of the analysis technique proposed here, based on functions of the digital imagery software eCognition professional 4.0, provides an objective and effective method for the assessment of fungal diversity in the context of environmental screening projects. It is demonstrated that strains of cultivated fungi can be quantitatively segregated with regard to specific false-color patterns, which reflect even the merest differences in pigment composition, indicating genotypic or phylogenetic disparities. Due to resolving subtle differences of phenotypic traits, a rapid recognition of (duplicate) genotypes is possible which allows the direct inference of the mycobial diversity of given environmental samples and a semi-quantitative or qualitative estimation of the fungal community structure. Two sets of image data from cultures were used in the current study: a minor set being applied for the definition of color classes and for usage in an image reference array, and a second, extended dataset for method validation. An objective assignment, based on false-color classification, was carried out by cluster analysis. High reproducibility using standardized methods makes this design an effective pre-screening option in the field of microbial environmental research. The application of false-color imagery may therefore be applied in fungal monitoring studies as a meaningful procedure supplementing molecular analyses by the identification of new strains irrespective of their relatedness.

Keywords Computer-aided identification · False-color imagery · Filamentous fungi · Morphological segregation · Phenotypic classification

A. Pietrowski (✉) · F. Flessa · G. Rambold
Lehrstuhl für Pflanzensystematik, Universität Bayreuth,
Universitätsstrasse 30 NW I,
95440 Bayreuth, Germany
e-mail: andrea.pietrowski@uni-bayreuth.de

Introduction

The number of fungal species assumed to exist worldwide ranges between 0.5 and 9.9 million, and might be as many as, conservatively estimated, 1.5 million (Hawksworth 2001, 2004). This high number is in accordance with the experience obtained from biodiversity inventory projects, where mostly high numbers of isolates are needed for assessing the factually present diversity of the fungal taxa or genotypes. Molecular techniques for assessing microbial community profiles applied in recent years are generally based on PCR amplification, coupled with DNA analysis methods (reviewed in Anderson and Cairney 2004). With next generation techniques coming along, the DNA chip technology has been adopted for achieving first estimates of composition and structure of fungal and bacterial communities (Ye et al. 2001; Gilbride et al. 2006; Malik et al. 2008; Truu et al. 2009).

For studying fungal diversity, direct DNA extraction is actually favoured because such an approach shows great promise for elucidating fungal community composition and shifts, e.g., in relation to edaphic conditions, environmental stress, or vegetation type (van Elsas et al. 2000; Lowell and Klein 2001; Chen and Cairney 2002; Anderson et al. 2003). However, molecular analyses of directly extracted fungal DNA and traditional, morphochemical analyses of samples taken into cultured strains may reveal strong discrepancies in resulting taxonomic spectra (Anderson and Cairney 2004; Bougoure and Cairney 2005). As both approaches obviously cover just segments of the entire fungal community present, neither can be considered as obsolete. Despite all the benefits from the techniques of direct DNA isolation from a substrate, cultivation of isolated fungi is therefore still important and also necessary for all purposes of applied research, e.g., on fungal metabolism.

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To use genomic sequence methods in monitoring projects and to avoid multiple sequencing of genetically identical isolates, a preselection mechanism for recognizing unidentified isolates like duplicates of already genotyped samples is considered necessary. Microscopic investigation is ineffective for that purpose as such an approach is relatively time-consuming, and diagnostically relevant characters, like conidial structures, do not always form under culture conditions, nor are they easily recognizable. Preselection methods based on the separation and identification of chemical compounds would also be possible, but are unsuitable for routine screening of high numbers of cultures. HPLC or mass spectrometry, providing highly resolved chemical profiles, may be considered a suitable high throughput option, but in view of the temporal investment for data evaluation, their application seems suitable only for approaches using pre-identified sets of samples.

To identify microbial taxa, several recently established high throughput level molecular techniques exist, like cyclic-array sequencing (Ronaghi 2001; Fakhrai-Rad et al. 2002; Doostzadeh et al. 2008; Eriksson et al. 2008; Gharizadeh et al. 2008). Such solutions, however, are still cost-ineffective. To effectively classifying (duplicates of) environmental fungal samples, representing a wide range of hitherto unknown genotypes, an identification approach based on digital imagery seems to be an adequate option. By extracting a maximum of information from macromorphological traits of fungal cultures, it serves as a suitable alternative. Colony growth speed, color zonation, and surface texture estimation are normally assessed visually and often result in highly subjective data recording. In a long-term monitoring study, e.g., of soil microbial communities, with a focus on seasonal or successional changes, automated computer-aided image array analysis proved to be a suitable objective method for the rapid classification and recognition of isolates. First essays of computer-aided identification of bacterial colonies were most promising (Liu et al. 2001; Ogawa et al. 2005). With regard to fungal colonies, such methods were applied only for identifying strains from monophyletic groups, e.g., *Penicillium* (Dorge et al. 2000; Hansen et al. 2003). However, classification and identification of fungal colonies derived from highly diverse natural microbial communities have hitherto rarely been accomplished by image analysis. Watrud et al. (2006) compared methods for estimating fungal species diversity using taxonomic, colony morphotype and PCR-RFLP methods and found traditional taxonomic identification corresponded by 78% with the molecular approach. They already showed that quantitative colony morphotyping can be used as a viable primary screening method, but to obtain high reproducibility of results, control of fungal colony morphotypes by applying specific culture media and standardized culture conditions was found to be an important precondition.

We here present the experimental design of a procedure for the identification of fungal strains obtained from a highly diverse microbial community to identify culture morphotypes using false-color classification. Based on the analysis of the spectral information in the RGB color channels, highly resolved image files of fungal cultures were analyzed using functions of the image analysis software eCognition Professional 4.0 (Definiens, Munich, Germany). The aim of our study was to develop an alternative approach to identifying and preselecting phenotypically corresponding duplicate fungal strains and, in this way, to considerably diminish the number of genotypically identical strains before starting subsequent procedural steps of DNA sequencing.

Materials and methods

Experimental design

The proposed approach for increasing the efficiency of fungal diversity studies combines molecular and image-guided analyses (Fig. 1). For a first setup, a subset of macro-morphologically distinct fungal colonies from environmental samples is randomly selected. At this point, classification is based on ‘natural color’ differences and color zoning under daylight (Fig. 1a). By use of ITS-nrDNA data for an assignment of the fungal strains to taxonomic levels, an initial reference dataset for subsequent image-guided analyses of new sets of unidentified fungal strains is established. To obtain strain-specific false-color patterns, digital images of the reference set are mounted into a combined ‘reference image array’. The assignment of colony surface details to object classes by visually ‘sampling’ disparate colony color zones or areas (‘determination of false-color classes’), and subsequent application of the obtained classes (‘classification’) allows for a phenotypic differentiation of genotypically different strains (‘false-color phenotype’).

Extended color class libraries are established by repeated procedural loops of defining additional false-color phenotypes (Fig. 1b), followed by an ‘application of reference classification’. For this purpose, a newly assembled ‘random image array’ comprising images of unidentified fungal strains along with a set of reference strains, is subjected to color classification based on the previously sampled classes. The false-color class records per colony are combined in a (0,1)-matrix (fungal strains × false-color classes) and transformed into a ‘false-color class dissimilarity matrix’ by using the Jaccard index hierarchical cluster analyses in Primer 6.0 (v. 6.1.6) with the group average

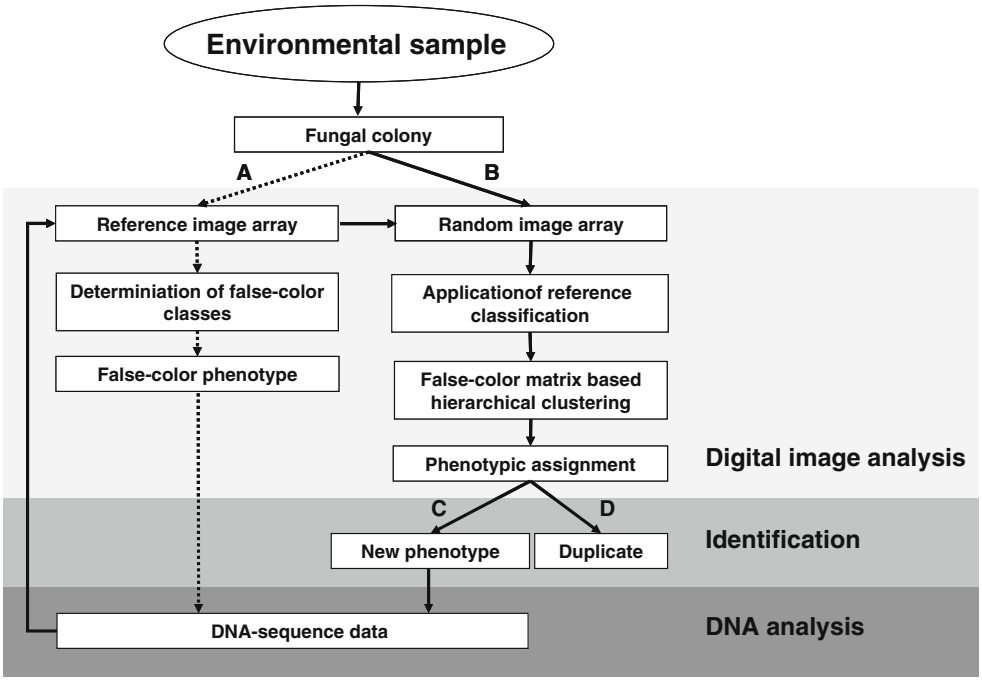

Fig. 1 The experimental design combines molecular and computer-based macro-morphological data ascertainment. A first reference image array (*broken line*) is combined with a subsequent random image array (*continuous line*) for effective diversity study. Basic molecular and image-array data complement one another along with increasing data volumes. A detailed description is given in the text

option being selected. This 'phenotypic assignment' allows for the recognition of new or duplicate, i.e. already recorded, phenotypes. For calibration of false-color pattern similarity, defined threshold values (Fig. 1c, d) can optionally be applied, but in the present study only strains with 100% similarity are regarded as identical in the sense of being genotypically equivalent. This strict approach of the false-color phenotypic assignment allows a most rapid recognition of duplicates (Fig. 1d). All colonies of less than 100% similarity to any reference strain are subjected to subsequent DNA sequence analysis, and thereafter inserted in the extended reference dataset to serve as additional reference during subsequent loops of the procedural steps described above. In this way, the number of fungal strains to be used as reference for subsequent analyses continuously increases, which also improves matching during study progression.

Fungal isolates used for method evaluation

For the present experimental study, the diversity of leaf-inhabiting fungi of *Calluna vulgaris* (L.) Hull. ('heather') was exemplarily examined. To extract the various fungal strains, leaves were surface-sterilized and put on yeast malt agar (YMA contains 4 g glucose, 10 g malt extract, 4 g yeast extract, and 12 g agar per liter) containing tetracycline 0.1% to suppress bacterial growth. Fungal isolates gained from the plant tissue were subjected to DNA analysis and yielded a total of 21 ITS nrDNA genotypes. In order to evaluate the success of the detection of identical genotypes by using eCognition software, each strain was represented by 3 replicates, resulting in 63 false-color images to be used for testing the approach. Strains are cultured on YMA in Petri dishes of 5.5 cm diam. For improved comparability of morphological variance due to developmental effects or different nutritive conditions, strictly defined culture conditions were applied. Cultures were incubated for 14 days at room temperature and constant lighting before digital images were taken.

In order to evaluate genotypic variation among nrDNA-ITS sequences, pairwise similarities were previously achieved by applying the stand-alone BLAST application 'blastall' (v. 2.2.18; ftp://ftp.ncbi.nih.gov/blast/executables/release/2.2.18/blast-2.2.18-ia32-win32.exe) on a FASTA database, containing all nrDNA-ITS sequences, with the parameters ' –m 8 –r 2 –G 5 –E 2'. The resulting dataset was transformed in a dissimilarity matrix by using the function 'simMatrix' in the R script 'RFLPtools' (Flessa et al. 2010). A hierarchical cluster analysis, based on this pairwise ITS dissimilarity matrix, was performed by applying the function 'hclust' (hclust, base R package stats; R Development Core Team 2010) with the methods 'single', 'complete', and 'average linkage'. Clusters with minimal similarities of 90% by at least one of the three methods were preliminarily grouped, using the R function 'cutree'. Sequences of each group were aligned and truncated at the ends to the length of the respective shortest sequence. The adjustment of sequence lengths served to avoid inconsistencies in the similarity values caused by sequence parts that were obtained from only a subset of the group. Subsequently, the pairwise similarities were recalculated as described above among the length-adjusted

sequences. The cluster analyses were repeated on this secondary similarity matrix and clusters were defined by cutting the resulting dendrogram at a height of 94–99% by the function 'cutree' (cutree, base R package stats) (Peršoh et al. 2010).

Fungal taxonomy applied follows Index Fungorum (2010, January). nrDNA-ITS sequences were used to determine the most similar sequences deposited in GenBank at the NCBI website (http://www.ncbi.nlm.nih.gov; status: 2010, January) using 'Mega BLAST' (Zhang et al. 2000) for obtaining taxonomic data. Sequences with highest percentual similarity found were taken as basis for assigning a preliminarily name to the isolates, following the concept of name assignment recommended by Peršoh et al. (2010).

Photography and pre-processing of images

RGB-spectral images of cultures of filamentous fungi were acquired 14 days after inoculation with a digital camera (Nikon D2x, Micro-Nikkor 60 mm F/2.8 D lens) mounted on an aluminum frame construction 36.5 cm above surface. Two neon lamps (L18 W/19 Daylight 5000 de Luxe) inclined 80° to the frame provided reproducible lighting conditions. A color chart (RAL 7005-HR mousy; German Institute for Quality Assurance and Certification e. V.) was used as standard background. Images were taken with a lens aperture of f/16, sensitivity ISO 100, balance preset d-0, color mode III (Adobe RGB), and a shutter speed of 0.5 s. For further processing, the Nikon electronic format NEF (nearly uncompressed) pictures were converted into JPEG (8-bit) format (JPEG quality: fine, JPEG compression: optimal compression, size 4,288×2,848 pixels). The single images were mounted to composed arrays of image files with Adobe Photoshop CS2 software (v. 9.0) using the contact sheet II preset. Before composition, the image sizes of the individual images were standardized (1,000×1,000 pixels). Subsequently, image analysis was performed by using eCognition professional software (v. 4.0).

Automated segmentation of digital images

In contrast to various other imaging systems, the eCognition image analysis is not based solely on pixel values but works with so-called 'segments' generated from areas of adjacent pixels. By selecting the parameter 'multiresolution segmentation', such areas are separated with regard to image contrast parameters, and thereby unit numbers for classification are dramatically reduced. For the present study, this method of automated segmentation was performed at one level, using the default homogeneity criterion values of 0.5/0.5 for color/shape, 0.20 for smoothness/compactness, and a scale parameter of 50.

Sampling of false-colors for establishing a color class library

The 'standard nearest neighbor' option using 'layer mean values' from RGB is selected to establish a false-color class library ('standard nearest neighbor' is a classifier being used to classify image objects based on given sample objects within a defined feature space). The channels for the gray-scale values, i.e. the brightness of the three RGB color channels, are set per default. False-color classes were defined by manually selecting segmented areas, resulting from preceding fragmentation. In this way, about 5–10% of the colony surface was sampled. The procedure was performed for each genotyped fungal reference colony, with every class being represented by its own false-color (reference image array; Fig. 3 I, II).

Application of false-color classification

After applying the false-color classes, i.e. classifying the composed (first or primary) reference image array, fungal colony images yield individual false-color patterns, representing 'false-color phenotypes'. Each of those phenotypes by definition includes only representatives of 100% pattern similarity. When applying the same (first or primary) set of class definitions to a 'random image array', which includes unidentified colonies along with the primary set (Fig. 1), strains with identical segment properties are identical with regard to their false-color phenotype and therefore considered most likely being genotypically identical. Cultures with deviating false-color patterns, however, are presumably different from the genotyped strains of the first set. After genotypic characterization by sequencing, such strains may be used as a reference in subsequent analyses and added to the next image array generation according to the procedures as outlined for the first array.

Statistical analyses of false-color patterns

The principle of the method is based on evaluating the color information present in the arrays after applying the automated classification by eCognition software. The similarity was evaluated as a distance between the observed false-color patterns. Detected false-color per colony as well as eventual dominance of certain false-color were coded as binary values in a data matrix. Incidental light reflections on the images were rare and ignorable. The similarities were calculated applying Jaccard index hierarchical cluster analyses by Primer 6.0 (v. 6.1.6) with the group average option being selected.

Results

Molecular characterization of fungal strains

21 fungal strains, corresponding with 21 genotypes, were used for the experiment. Each of the fungal strains were represented by three additional replicates, resulting in 63 false-color images. In a first step, nine of the isolates were randomly chosen for the first reference image array (Table 1). In the subsequent procedural 'loop', the remaining strains and their replicates were analyzed and either identified as a new strain (and therefore to be used as a new reference) or recognized as a known false-color phenotype. According to Mega BLAST search results and hierarchical cluster analysis, sequences belonged to 12 different taxa, 4 of them being represented by more than one strain (Table 1). The dendrogram of ITS sequence dissimilarities shows that sequences resulting as representing one and the same taxon cluster together significantly (Fig. 2). Due to the high congruency of taxon names and sequence similarities, taxon names are referred to when discussing the false-color phenotype–genotype correspondence.

Identification of fungal colonies by false-color phenotype comparison

Genotyped fungal strains were used as references to ensure a sampling of taxa of different phylogenetic relationships. Digital images of the reference cultures were mounted to a reference image array in order to define the initial class hierarchy. The application of the false-color classification onto the image array resulted in specific false-color patterns of the individual reference cultures (Fig. 3III). Hierarchical clustering displayed the image group similarities and confirmed segregation at species level as expected (Fig. 4). All fungal strains used as reference cultures corresponded to one false-color phenotype.

The distinction between the preset false-color phenotypes and fungal strains acting as new references (Fig. 4) also worked for the random image array (Fig. 5). As shown in Fig. 2, fungal strains assignable to the same taxon names by Mega BLAST searches in GenBank corresponded with a taxon-specific false-color phenotype. In detail, false-color pattern identity was given for strains identified as being conspecific by Mega BLAST results, as shown for 'Leotiomycetes sp.' (AP288, AP404), *Glomerella* sp. (AP412, AP730), and 'Capnodiales sp.' (AP687, AP348, AP499). Due to such a high degree of accordance between obtained taxon names and false-color phenotypes, the false-color phenotype could be recognized as successfully defined and identified and, in the case of dual or threefold matches of the respective taxon name, its recognition can be considered as being significant. The sensitivity of this method worked excellently with regard to genus *Glomerella*. The dataset included several genotypes assigned to that genus, i.e., AP412, AP730, AP437, and AP480. However, strains identified in this study as belonging to a genus of its own displayed different false-color phenotypes (AP328) (Figs. 2 and 4).

The present dataset also included a couple of fungal strains which were assigned to *Sirococcus conigenus* (AP323, AP424, AP500, and AP508). As these strains substantially differ from each other even in their natural color patterns (Fig. 5), the automated assignment resulted in additional false-color phenotypes (P02, P14–P17; Figs. 2 and 4).

Detection of identical genotypes

The present approach proved to be a precise method for the identification of genotype- corresponding false-color morphotypes (Fig. 4). The replicates of 18 different strains, which are not always easily to distinguish with respect to their natural colors, could be successfully separated according to their false-color patterns with a similarity match of 100%. However, representatives of the poorly pigmented cultures AP505 ('Pleosporales sp.') and AP288 ('Leotiomycetes sp.') were not assigned to the corresponding reference cultures (Fig. 4). Despite their growth under identical culture conditions, coloration within one genotype may be somewhat variable and is not a fully reliable marker for genotypic identity. Morphologically just superficially similar (but genotypically different) strains are distinguishable after applying a false-color class hierarchy as shown in Fig. 4 for AP288 ('Leotiomycetes sp.') and AP412 (*Glomerella* sp.). Both strains clearly result in different taxon names by Mega BLAST search despite both sharing a bright creamy-colored colony surface.

Discussion

Identification of fungal colonies by false-color phenotype comparison

In the case of strains that substantially differ from each other even in their natural color patterns (i.e. *Sirococcus conigenus* AP323, AP424, AP500, and AP508; Fig. 5), the automated assignment resulted in additional false-color phenotypes (P02, P14–P17; Figs. 2 and 4). Their recognition appeared to be more problematic due to the secretion of secondary compounds into the growth medium. In order to consider agar coloration as a decisive color character, the criterion of medium layer thickness has to be standardized. A synchronization of the metabolite production is difficult to realize due to its dependence on various parameters like

Table 1 Fungal strains used in this work were isolated from leaves of *Calluna vulgaris* plants

Closest species match by Mega BLAST search[a]					Name assignment					
Genotype ID	GenBank Acc. No.	Deposited as	Bit-Score	e-value	Assigned name (="consensus name")	Number of sequences	Outliers	Environmental samples	Lowest Bit-Score	Highest e-value
AP421	EU167592.1	*Cladosporium* sp.	900	0	'Capnodiales sp.'	3	0	0	900	0
AP499	AM999588.1	'Mycobionta sp.'	870	0	'Capnodiales sp.'	3	0	1	793	0
AP412	EU482289.1	*Colletotrichum* sp.	926	0	*Glomerella* sp.	77	2	21	905	0
AP437	AY376530.1	*Colletotrichum crassipes*	915	0	*Glomerella* sp.	78	3	19	894	0
AP459	AB233343.1	*Glomerella graminicola*	929	0	*Glomerella* sp.	19	1	0	857	0
AP288	FJ904499.1	*Allantophomopsis* sp.	773	0	'Leotiomycetes sp.'	25	5	8	732	0
AP505	AY805589.1	*Phoma herbarum*	821	0	'Pleosporales sp.'	80	4	16	776	0
AP658	AY251089.2	*Mycosphaerella iridis*	865	0	'Pleosporales sp.'	78	1	21	889	0
AP323	AY168972.1	*Sirococcus conigenus*	802	0	*Sirococcus conigenus*	7	2	2	767	0
AP460	EU167602.1	*Mycosphaerella harthensis*	802	0	'Capnodiales sp.'	2	0	0	802	0
AP488	GU214651.1	*Sphaerulina polyspora*	863	0	'Capnodiales sp.'	85	0	15	863	0
AP348	AM999588.1	'Mycobionta sp.'	811	0	'Capnodiales sp.'	3	0	1	806	0
AP687	AM999588.1	'Mycobionta sp.'	822	0	'Capnodiales sp.'	3	0	1	811	0
AP328	EF592089.1	'Mycobionta sp.'	926	0	'Mycobionta sp.'	36	0	31	854	0
AP480	DQ003101.1	*Glomerella acutata*	953	0	*Glomerella* sp.	99	1	0	942	0
AP730	EU482289.1	*Colletotrichum* sp.	942	0	*Glomerella* sp.	74	1	25	922	0
AP404	EU625294.1	'Mycobionta sp.'	817	0	'Leotiomycetes sp.'	16	1	4	797	0
AP424	AY168971.1	*Sirococcus conigenus*	821	0	*Sirococcus conigenus*	7	2	2	649	0
AP436	FM172748.1	'Mycobionta sp.'	850	0	*Sirococcus conigenus*	8	2	2	782	0
AP500	AY168972.1	*Sirococcus conigenus*	804	0	*Sirococcus conigenus*	7	2	2	778	0
AP508	FM172748.1	'Mycobionta sp.'	817	0	*Sirococcus conigenus*	6	2	2	787	0

[a] Taxon names obtained by best Mega BLAST search matches in GenBank (www.ncbi.nlm.nih.gov) are listed according to their use as reference in the reference image array (Fig. 3, see below) in the upper part, and the additional strains in the lower part. Sequences with highest similarity found were taken as basis for assigning a preliminarily name to the isolates, following the concept of name assignment recommended by Peršoh el al. (2010) and are listed alphabetically

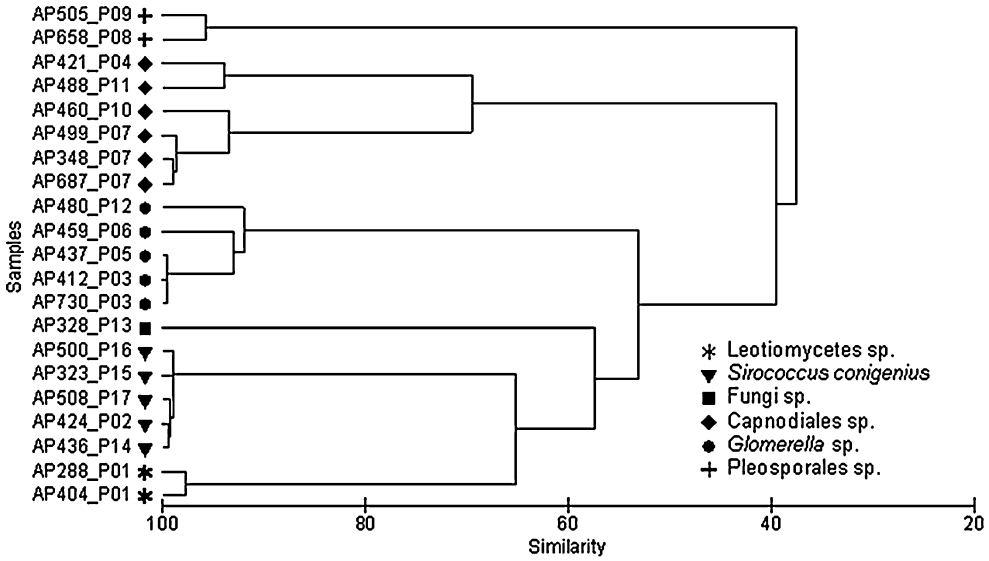

Fig. 2 Hierarchical cluster analysis (average linkage) of ITS-nrDNA sequence data of endophytic fungi of *Calluna vulgaris*. *Numbers* following strain names (*P*) indicate phenotypic classification, name assignment due to Mega BLAST search is coded in *symbols*. A pairwise similarity matrix of the ITS 1 and ITS 2 regions of the nrDNA was used for revealing genotypic variation of the fungal strains. The phenotypic classification has been received after digital image analyses

acidity and glucose contents of the medium. Under unequal conditions, even genotypically identical strains may produce deviant types and concentrations of secondary metabolites (Larsen et al. 2005). However, a verification of species identification by alternative identification methods would be of special interest for those cultures which differ from each other even in their natural color patterns.

In the present study, the ITS nrDNA identities of the strains were determined by comparison with published sequences, but it should be considered that identical names resulting from Mega BLAST are not necessarily markers for identical genotypes (Peršoh et al. 2010). Therefore, the application of threshold values for both sequence similarity and false-color phenotype assignment are essential to give support to the reliability of identification results. Genotypic variation would have to be revealed by detailed sequence alignments and subsequent phylogenetic evaluation. ITS sequencing was used just to demonstrate exemplarily genetic identity. Certainly, the application of beta tubulin and other phylogenetic markers is possibly more suitable for certain fungal groups of different relationships. However, in the present study, the ITS rRNA gene proved to be sufficient for proofing genetic identity of the false-color groups and additional markers were not mandatory.

Detection of identical genotypes

The present approach proved to be a precise method for the identification of genotype corresponding false-color morphotypes (Fig. 4). However, representatives of the poorly pigmented cultures AP505 (‘Pleosporales sp.’) and AP288 (‘Leotiomycetes sp.’) were not assigned to the corresponding reference cultures (Fig. 4). Despite their growth under identical culture conditions, coloration within one genotype may be somewhat variable and is not a fully reliable marker for genotypic identity.

Nevertheless, minor differences in periodic zoning, for instance, may cause differences in their phenotypic classification. However, this aspect is a mostly peripheral

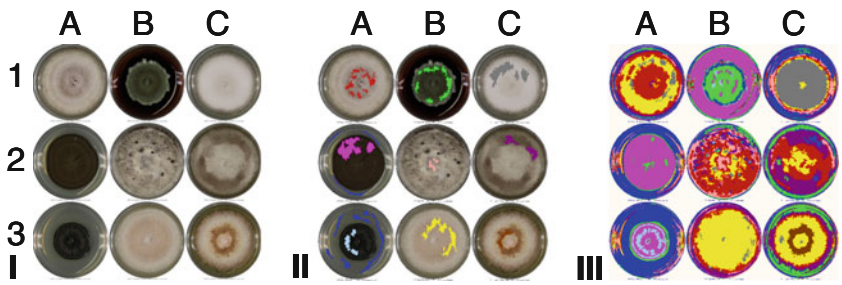

Fig. 3 Reference image array include fungal colonies used as references for subsequent environmental screening. For the detailed position of the isolates, see Table 1. *I* Composed natural color image; *II* Sampling of false-color areas (one false-color per culture as well as pure agar (A3) for definition of the class hierarchy), *III* Resulting false-color patterns after application of the class hierarchy by using standard nearest neighbor classification. *A1* AP288_‘Leotiomycetes sp. ’, *A2* AP421_‘Capnodiales sp.’, *A3* AP499_‘Capnodiales sp.’, *B1* AP323_*Sirococcus conigenus*, *B2* AP437_*Glomerella* sp., *B3* AP505_‘Pleosporales sp.’, *C1* AP412_*Glomerella* sp., *C2* AP459_*Glomerella* sp., *C3* AP658_‘Pleosporales sp.’

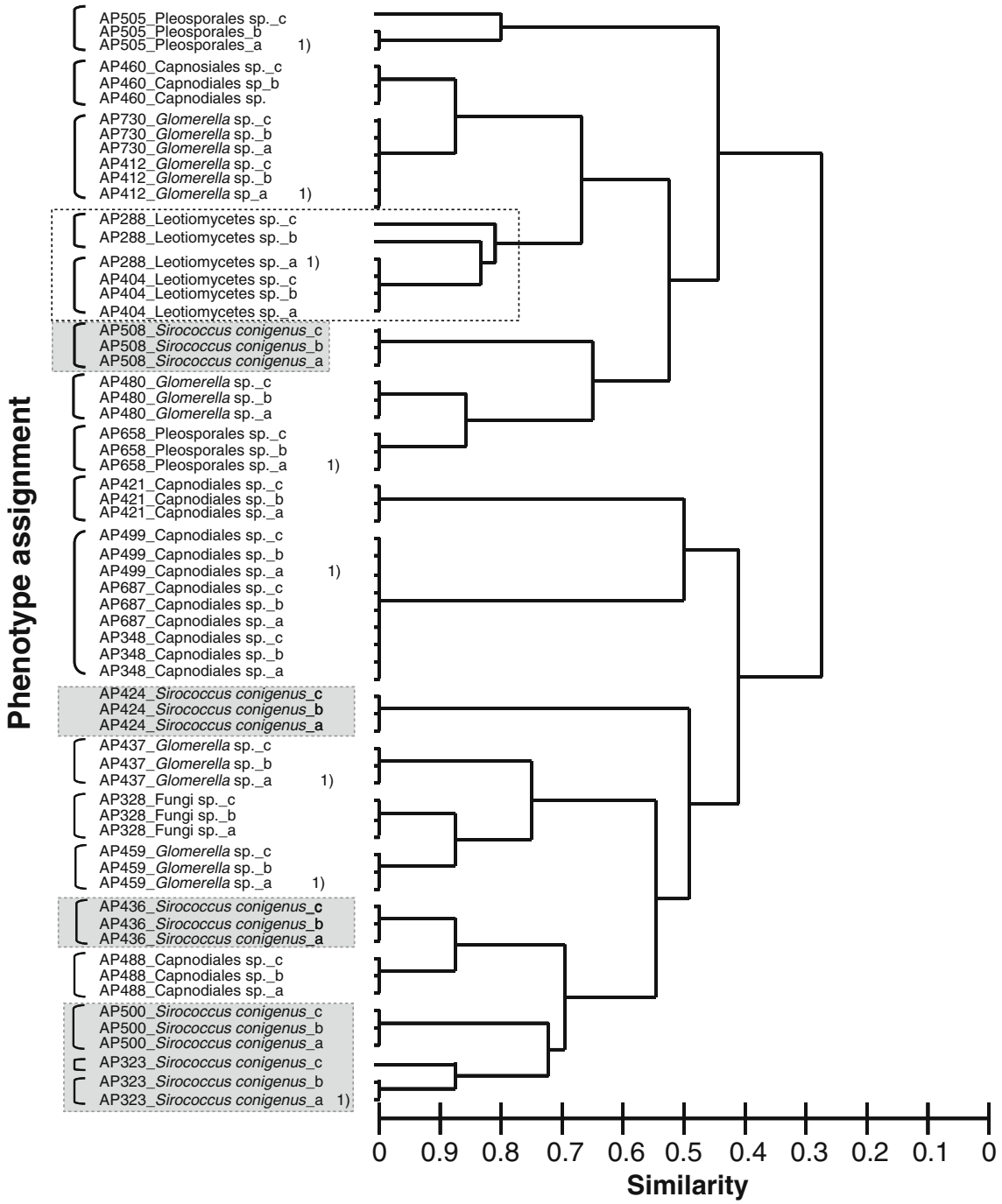

Fig. 4 Results of false-color matrix based hierarchical clustering in the random image array. A similarity of 100% indicates a specific false-color phenotype (*a–c* indicates replicates of each genotype-ID). 1) Sampled phenotypes act as reference colonies in the reference image array. Fungal strains were assigned to available references, i.e. false-color phenotypes or new false-color phenotypes, acting as additional references in subsequent monitoring campaigns were identified. Different genotypes of *Sirococcus conigenus* were assigned to individual phenotypes according to different macro-morphological pigmentation structures (marked in *gray*). Different genotypes of 'Leotiomycetes sp.' were split in two phenotypes as a consequence of 'over-sensitivity' (*broken line*)

problem and just depending on the specific growth mode of the fungal colony. Morphologically just superficially similar (but genotypically different) strains are distinguishable after applying a false-color class hierarchy as shown in Fig. 4 for AP288 ('Leotiomycetes sp.') and AP412 (*Glomerella* sp.). Both strains clearly result in different taxon names by Mega BLAST search despite both sharing a bright creamy-colored colony surface. This means that the

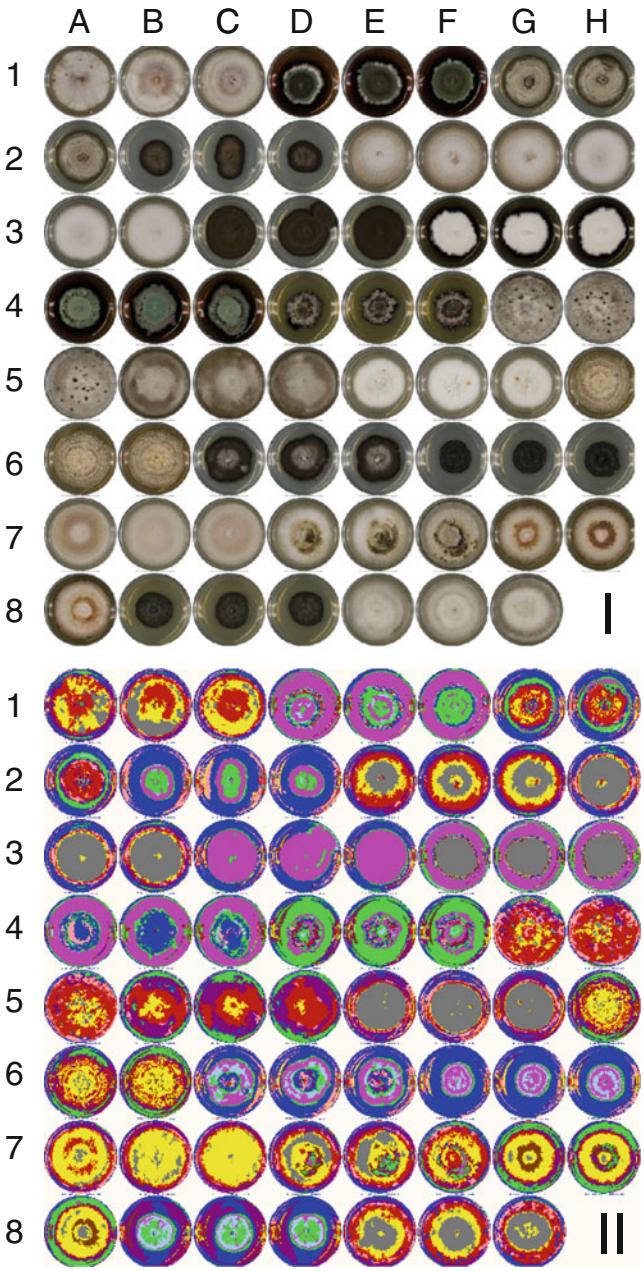

Fig. 5 Random image array include the total set of fungal colonies. *I* Natural-color images; *II* result of a standard nearest neighbor classification. *A1, B1, C1* AP288_'Leotiomycetes sp.'; *D1, E1, F1* AP323_*Sirococcus conigenus*; *G1, H1, A2* AP328_ 'Fungi sp.'; *B2, C2, D2* AP348_'Capnodiales sp.'; *E2, F2, G2* AP404_'Leotiomycetes sp.'; *H2, A3, B3* AP412_*Glomerella* sp.; *C3, D3, E3* AP421_'Capnodiales sp.'; *F3, G3, H3* AP424_*Sirococcus conigenus*; *A4, B4, C4* AP500_*Sirococcus conigenus*; *D4, E4, F4* AP436_*Sirococcus conigenus*; *G4, H4, A5* AP437_*Glomerella* sp.; *B5, C5, D5* AP459_*Glomerella* sp.; *E5, F5, G5* AP730_*Glomerella* sp.; *H5, A6, B6* AP480_*Glomerella* sp.; *C6, D6, E6* AP488_'Capnodiales sp.'; *F6, G6, H6* AP499_'Capnodiales sp.'; *A7, B7, C7* AP505_'Pleosporales sp.'; *D7, E7, F7* AP508_*Sirococcus conigenus*, *G7, H7, A8* AP658_'Pleosporales sp.'; *B8, C8, D8* AP687_'Capnodiales sp.', *E8, F8, G8* AP460_'Capnodiales sp.'

extreme sensitivity of the method allows for the differentiation between morphologically nearly identical cultures.

However, the focus of this paper was to show that genetically identical strains (gathered during field studies) can be recognized by image analysis. It was *not* the aim of the study to *identify* strains, which is achieved by doing traditional light microscopic identification and/or by applying one or various gene(s) for doing Mega BLAST search at GenBank. In the present study, genetically analysis was just done to prove the reproducibility of the proposed false-color classification, but not to identify taxa.

Possible limitations of the method

The high diversity of fungal genotypes results in a high variation of pigmentation of the fungal culture. It is evident that the similarities within and the differences between the macroscopic coloration of the various genotypes, represented by specific combinations and patterns of classes, correlate with the type, concentration and combination of specific pigments being present. It is known for many fungal groups that the combination and concentration of secondary metabolites basically reflect phylogenetic units at the species level and below. Characteristic pigmentation patterns of fungal cultures reflect specific secondary metabolism pathways, and therefore act as chemotaxonomic markers. Pigments and other secondary metabolites have therefore traditionally been used in taxonomy of teleomorphic and anamorphic fungi (Frisvad et al. 1998; Stadler et al. 2003), frequently resolving even better between closely related taxa than do molecular markers like ITS nrDNA (Skouboe et al. 1999).

The sampling procedure for creating a false-color class library is the only decisive and therefore subjective procedural step in the whole process. It could be shown in the present study that the sampling of one color segment per culture led to a sufficiently high dissimilarity for classification. With increasing data volume, the number of false-color classes also increases, and differences based on one color segment become less relevant. Simultaneously, the number of available false-colors decreases and it therefore seems reasonable to keep color sampling per culture to a minimum.

Any changes in the composed array, e.g., adding new reference cultures and/or new false-colors, entail an updating of classification results. This makes a recalculation of the statistical hierarchical analyses, based on an ordinary presence/absence matrix of detected false-colors, necessary. Although the dominance or cover of the respective colors is already considered in the present approach, an additional relative weighting of occurring false-colors, supported by calculative statistics, would possibly improve the results even more. The analyses as performed here disallow a true

estimation of the proportion of the individual colors. Small amounts are overrated and may disproportionately affect the similarities to some degree. For this reason, culture and photographing should be standardized to a maximum extent. Further automation of the mechanical and software-based procedural steps would enhance the effectiveness of the method described here.

The very high sensitivity of the method allows for the differentiation of most similar cultures. Therefore, morphologically variable strains are represented by multiple reference colonies. This 'oversensitivity' may sometimes be crucial, but at least does not entail wrong assignments. A separation of fungal colonies into homogenous units is always successful, but their assignment not always complete.

Conclusion

In monitoring campaigns with a focus on microbial communities, automated computer-aided image array analysis proved to be a suitable and objective method for rapid recognition and classification of fungal isolates. We developed an approach for *preselecting* false-color phenotypically identical fungal strains and in this way to considerably diminish the number of correspondingly genotypically identical strains before starting subsequent procedural steps of DNA sequencing. First approaches of computer-aided identification of bacterial colonies were most promising (Liu et al. 2001; Ogawa et al. 2005). In the present study, a standardized method for an objective analysis and classification of fungal isolates was elaborated. For growth under standardized conditions, suitable media were chosen. In addition, standardized illumination conditions were established for obtaining reproducible digital images. Digital photographing and processing of images into false-colors minimized the subjectivity of the operator.

The method is suitable for routinely testing cultures with regard to genetic identity, and also for eliminating duplicates during the screening for new strains. The easy recognition of misidentifications and of contaminations by applying it as a standard curatorial measurement for the verification of strain identity may also be of special interest for institutional culture collections.

Despite single inconsistencies with regard to certain strains, reproducibility of the method can be taken for granted. False-color analyses provide a much higher resolved classification compared to natural color images by clearly reflecting even minor nuances of color patterns for the class hierarchy. In the frame of long-term monitoring campaigns taking during extensive time periods, e.g., with focus on seasonal influence or successional changes, similar morphologies of colonies frequently occur. As the percentage of misidentification is sufficiently low, the method is suitable for preselecting relevant fungal strains for DNA sequence analysis in the frame of environmental screening projects encompassing a high diversity of different fungal genotypes and their corresponding phenotypes.

The proposed approach is especially suitable when fungal colonies exhibit mostly similar morphologies. These fungal strains can be segregated according to their false-color patterns, which reflect even the merest differences in pigmentation. It has to be tested, however, whether analyses are similarly successful when applied to much larger datasets.

Rapid genotyping of strains combined with an assignment of unclassified strains to new or already known false-color phenotypes by the use of digital images is apparently a suitable approach for preselecting fungal strains in culture. The advantages are in the much higher optical resolution in contrast to a visual computer-unaided estimation. The present approach is, therefore, a non-competing method for a preselection of identified or unidentified genetically identical strains.

Research costs are minimized by effective sequencing as well as by operating the main part of the working steps using standard image analysis software.

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Analysing diversity and community structures using PCR-RFLP: a new software application

FABIENNE FLESSA,* ALEXANDRA KEHL† and MATTHIAS KOHL‡
*Section Mycology, University of Bayreuth, 95440 Bayreuth, Germany, †Botanical Garden, University of Tuebingen, 72076 Tuebingen, Germany, ‡Department of Medical and Life Sciences, Furtwangen University, 78054 Villingen-Schwenningen, Germany

Abstract

Restriction fragment length polymorphism tools is an R application which supports a complete workflow of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), dealing with the problems which accompany analysis when PCR-RFLP is used in diversity studies. Large numbers of different RFLP samples obtained from multiple electrophoresis runs might lead to limitations or misidentifications due to the need for band matching in most existing software applications. Due to the common problem of variation in the density of bands (i.e. distances between bands or visual intensity) in the electropherograms, it is desirable to have options for handling samples with uncertain or faint bands. As a further step in the workflow, scientists often use DNA sequencing to identify individual genotypes, so that the use of specific software to combine these tasks might be helpful. With this background, we here present an application that supports a complete workflow, starting with the analysis of single species samples by PCR-RFLP, to PCR-RFLP genotype identification based on a reference data set and DNA sequencing followed by similarity analysis. RFLPtools is a freely available, platform-independent application which provides analysis functions for DNA fragment molecular weights (e.g. by RFLP analysis), including similarity calculations without the need for band matching. As it is written for the statistical software R, other statistical analyses might also be easily applied.

Keywords: diversity studies, R package, RFLP analysis, sequence analysis

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Introduction

In many biological disciplines, fingerprinting techniques such as restriction fragment length polymorphism (RFLP) have become useful and commonly applied laboratory tools in biodiversity research to recognize and analyse genotypic diversity. The RFLP technique, especially, is frequently used in population studies targeting various organisms such as plants (Tsarouhas *et al.* 2002) or symbiotic (Sykorova *et al.* 2007), pathogenic, or soil-inhabiting microbes (Watrud *et al.* 2006; Duran *et al.* 2009). Polymerase Chain Reaction (PCR)-RFLP is based on nucleotide differences that affect the binding site for restriction enzymes in certain DNA arrays, which after enzymatic digestion and amplification of the fragments lead to different fragment patterns following visualization by electrophoresis. Other related fingerprinting techniques are Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.* 1995), Random Amplification of Polymorphic DNA (RAPD), STRs (Single Tandem Repeats), microsatellites (Goldstein & Schlötterer 1999) and Terminal RFLP (T-RFLP) (Liu *et al.* 1997).

By comparing the resulting fragment patterns, the RFLP technique is capable of detecting and discriminating genotypes after a cloning step to gain single species samples from environmental samples or populations. To further characterize genotypes (or organisms) in such studies, PCR products of single RFLP samples can be DNA-sequenced and the resulting sequences further compared by nucleotide sequence alignment searches performed by specific algorithms, for example by the standalone Basic Local Alignment Search Tool (BLAST) (Zhang *et al.* 2000) or local BLAST v7.0.9 (Hall 1999), with published sequence data.

However, sample treatment and the analysis of fragment patterns are susceptible to technical difficulties if electrophoresis gels are used, which might affect and reduce the reproducibility and reliability of results. The processing of fragment patterns might be hampered, for example, by more or less serious lateral deformations of runs during electrophoresis (so-called 'smiley effects') or

Correspondence: Matthias Kohl, Fax: +49-7720-307-4207;
E-mail: Matthias.Kohl@stamats.de

by the presence of large amounts of highly diverse and sometimes unresolved or superimposed bands, which contribute to analysis and interpretation bias. Additionally, the comparability of large numbers of electrophoretic runs is sometimes limited, especially in biodiversity studies, where large amounts of samples are usually generated on numerous electrophoresis gels. In the analysis and comparison of the resulting fragment patterns, band matching is a precondition for subsequent analyses in most software applications and is often problematic due to the mentioned constraints. Furthermore, proprietary applications that provide adequate analysis functions are mostly rather expensive.

Comparisons of fragment sizes or peak heights are already implemented in free bioinformatic software applications such as GERM (Dickie *et al.* 2003), which is based on Visual Basic application macros, or FragMatch, a Java application (Saari *et al.* 2007). Table 1 summarizes the possibilities and limitations of these two applications compared with RFLPtools. Free software tools for other genome fingerprinting techniques also exist (e.g. Genographer, RawGeno, OptiFLP). However, tools for RFLP analysis disregard some of the specific problems which occur during the analysis of electrophoresis runs, such as biased electrophoresis fragment patterns and deformations during gel runs such as smiley effects. The problems often propagate as the number of samples analysed, and the diversity of band patterns increase.

Therefore, a free and platform-independent method for reading fragment pattern data derived from molecular fingerprinting analysis such as PCR-RFLP, without the constraints of matching bands in parallel runs, is required. Using the methods of matching bands, it is essential that there is no deformation in the electrophoresis gel. As soon as nonhomogeneous flow rates exist, it is difficult to define the exact distance for each fragment in the run. It is most probably that identical samples are interpreted as different RFLP types if the method of matching bands is applied to the run. Therefore, in RFLPtools, instead of band matching, distances are used for the analysis of the RFLP types.

Restriction fragment length polymorphism tools was mainly designed for data gathered from images of electrophoresis gels. Due to the common problem that band density and resolution might vary between individual gels, options to handle samples with doubtful or faint bands are desirable. To improve data quality, it is also necessary to have options for checking the reliability of the data.

Particularly for diversity studies with large and heterogeneous data sets, such analysis software should include a function for comparing fragment patterns with a reference data set comprising known fragment patterns of previously identified genotypes or organisms. Such an option should allow the fast matching of newly recognized samples with already characterized RFLP samples. A tool for the visual comparison of fragment patterns with reference data sets would help facilitate the rapid identification of unknown samples. For further processing of the data obtained, a suitable, freely available analysis application with a high degree of interoperability and flexibility and the option to read and write standard data exchange files for communicating with applications that provide additional statistical analysis methods is still needed.

Table 1 Comparison of GERM, FragMatch, and RFLPtools

	GERM	FragMatch	RFLPtools
System requirements	Microsoft Excel	Java	Independent
Based on	Spreadsheet formulas and macros, programmed in Visual Basic	Java	R
Quality check for input data	No	No	Yes
Different methods for analysis of RFLP fragment patterns	No	No	Yes
Method for analysis of RFLP fragment patterns	Band matching	Band matching	Distances
Methods for handling biased data	No	No	Yes
Methods for handling doubtful bands at a lower length threshold	Yes	No	Yes
Graphical visualization	Yes	Yes	Yes
Ability to compare new data with reference database	Yes	Yes	Yes
Designed for multiple data sets from a single sample	Yes	Yes	Yes
Function for comparing sequence similarities	No	No	Yes

Methods and implementation

Restriction fragment length polymorphism tools was developed to support the complete workflow, from the

analysis of biotic diversity after a cloning step, to obtaining single species samples from environmental samples by PCR-RFLP and to the identification of RFLP fragment patterns via sequencing. It includes several functions to import fragment pattern data, estimate their similarity and compare the resulting RFLP types with a reference data set for fast identification, as well as the possibility of a similarity analysis of DNA sequences.

In this section, we explain the functions and their use during a biodiversity study.

Restriction fragment length polymorphism tools, like many other R packages (R Development Core Team 2012), is command-line driven. It is installed by the command install.packages('RFLPtools', dependencies = TRUE). Functions are directly invoked by the user, in parts specified by arguments and options. Every session using RFLPtools in R starts with the command 'library (RFLPtools)', which loads all RFLPtools functions into the R environment. Information about the package (i.e. the DESCRIPTION file), as well as the list of included functions, can be displayed with the command 'library (help = RFLPtools)' (see also Table 2). There is a detailed help file (vignette), showing typical workflows which can be opened inside R by vignette('RFLPtools').

Before starting the analysis of fragment patterns via RFLPtools, fragments have to be detected using any graphical fragment pattern analysis software package, such as GeneProfiler (Scanalytics Inc.) or the free gel analysis macro MolWt (http://www.phase-hl.com/imagej.htm), which are able to generate simple text report files, including data on the molecular weight of detected bands, sample names and band numbers.

Table 2 Functions and their description

Name	Description
RFLPcombine	Combine multiple data sets from a single sample (i.e. separate digests with two or more enzymes)
RFLPdist	Compute distances for RFLP data
RFLPdist2	Compute distances for RFLP data where some bands may be missing
diffDist	Distance matrix computation based on successive differences
linCombDist	Linear combination of distances
RFLPdist2ref	Compute distance between RFLP data and RFLP reference data
RFLPlod	Remove bands below lower length threshold
RFLPplot	Plot RFLP data
RFLPqc	Quality control for RFLP data
RFLPrefplot	Function for a visual comparison of RFLP samples with reference samples
nrBands	Function to compute number of bands
read.blast	Read BLAST data
read.rflp	Read RFLP data
sim2dist	Convert similarity matrix to dist object
simMatrix	Similarity matrix for BLAST data
write.hclust	Cut a hierarchical cluster tree and write cluster identifiers to a text file

In contrast to existing applications, RFLPtools provides an optional quality check of the RFLP band patterns by comparing the sum of the molecular weights of all bands per sample with the given molecular weight of the complete PCR product within a certain range of tolerance (function RFLPqc). This option allows the identification of biased data and provides decision support for either excluding the respective samples from further analysis or for rechecking the doubtful band patterns on the original gel electrophoresis image. Such samples are detected due to the fact that a profile with an outlier sum would contain a fragment which would not have been generated by restriction of the PCR product or would have been subsequently contaminated.

With the function RFLPlod, which can be used to remove all bands below a given threshold, the package deals with uncertain or dubious bands of shorter lengths which may not be recorded or cannot be sized in a fraction of the samples.

Subsequently, the RFLP samples are grouped by fragment numbers (function nrBands), based on the assumption that only samples with identical band numbers could belong to identical genotypes. RFLPtools then computes distances between the fragment patterns of RFLP samples within each of these groups (function RFLPdist), based on the molecular weight of the bands, evoking the function dist (base R package stats) with default Euclidean distance. Alternatively, different distance methods such as 'Manhattan' or 'Canberra' or even completely different functions can be used. The use of migration distances of the molecular weights of fragments instead of band matching is the main advantage of RFLPtools. As RFLPtools generates objects of basic R data types, high compatibility with other R libraries and analysis methods are ensured, which is considered a clear advantage over other existing software solutions.

In cases where band detection is uncertain, for example, due to density variation between gels and samples, a second method of similarity analysis can be used (function RFLPdist2). This involves the computation of the distance between the molecular weight of a sample S1 with x bands and a sample S2 with x + y bands and the distances between the molecular weight of sample S1 and the molecular weight of all possible combinations of S2 with x bands are computed. The distance between S1 and S2 is then defined as the minimum of these distances.

Let us consider an artificial example: sample S1 has two bands with molecular weights m1 and m2 (m1 > m2); sample S2 has three bands with molecular weights n1, n2 and n3 (n1 > n2 > n3). RFLPdist2 computes the distance

between S1 and (n1, n2), (n1, n3), (n2, n3), and the minimum of the three distances is returned. Let us further assume that m1 = n1 and m2 = n3 then the distance between S1 and (n1, n3) is zero, which is also the minimum distance returned by RFLPdist2. There is also an option to set a lower limit, LOD. If LOD is specified, it is assumed that missing bands occur only below this threshold, that is, the number of bands larger than or equal to LOD has to be identical and all possible combinations are only considered for bands smaller than the LOD.

Furthermore, slight variation between gels and resulting differences in molecular weight detection between identical RFLP types, for instance due to smiley effects, can be anticipated using the functions diffDist and linCombDist. The function diffDist computes and returns the distances between the rows of a data matrix, where instead of the row values as in the case of 'dist', the successive differences of the row values are used. The function might be helpful if there is a shift with respect to the measured bands, that is, the Euclidean distance of (550, 500, 300, 250) and (510, 460, 260, 210) will be 0 instead of 80. Additionally, the function linCombDist was implemented, which uses a linear combination of distances and provides a way to combine molecular weights and band spacing to calculate the similarity of samples, with the possibility of choosing weights for both methods. With the help of linCombDist, two distance measures can be simultaneously specified to compute the distances between the rows of a data matrix. Depending on the chosen weights w1 and w2, a linear (w1, w2 arbitrary) or convex (w1 + w2 = 1) combination of the two results can be calculated.

In case multiple data sets from a single sample (i.e. separate digests with two or more enzymes) are available, the function RFLPcombine can be used to combine an arbitrary number of data sets.

Based on the calculated distances, hierarchical cluster analysis of RFLP samples can be performed using the function hclust (R base package stats), where the default clustering method is 'complete linkage'. Other clustering or unsupervised learning methods such as multidimensional scaling can also be applied easily. To obtain the final groups of identical samples, the value at which the dendrogram should be cut (cutree, R base package stats)

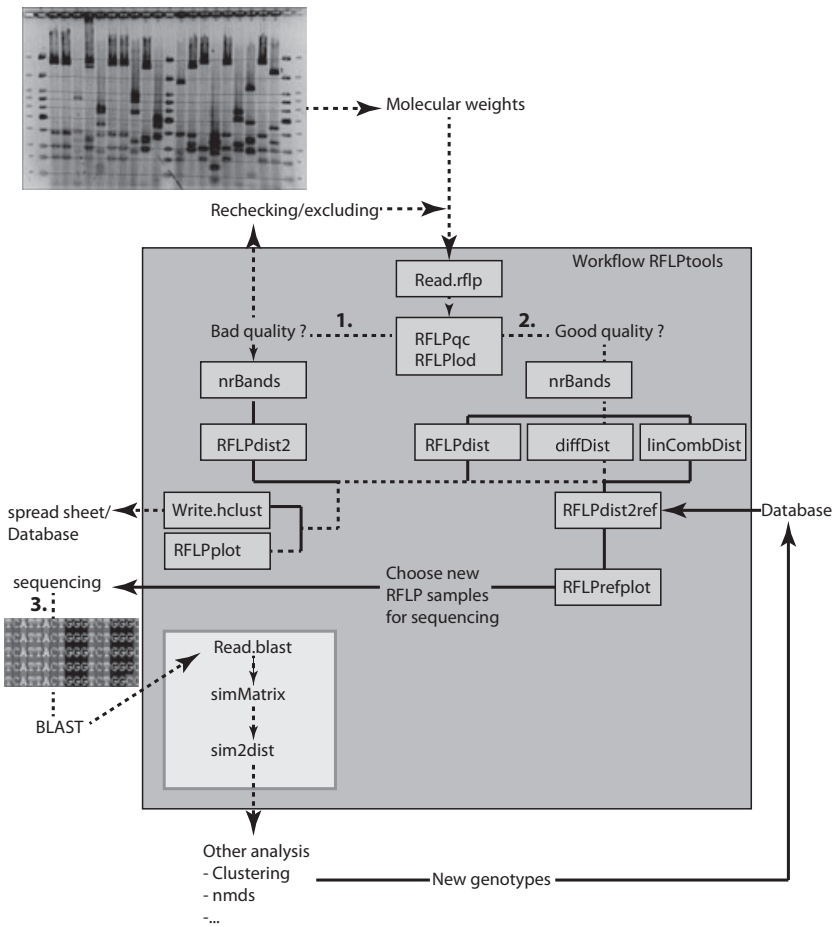

Fig. 1 Workflow of RFLP samples during diversity studies. The pale grey area summarizes functions included in R package RFLPtools. Dotted lines represent the workflow applied on the example data: 1. The RFLP input file contained manipulated samples, which were excluded after RFLPqc. 2. A new input file was analysed, an RFLPplot was drawn (see Fig. 2), and the results of clustering were exported using write.rflp. 3. Sequences of all RFLP samples in the example data were analysed.

must be considered by the scientist. Therefore, the similarity of samples (e.g. in terms of Euclidean distance) can be plotted using dendrograms and parallel molecular weight patterns, which enable a rapid and rather easy detection of identical samples (function RFLPplot) (see Fig. 1). After cutting the dendrograms (cutree, R base package stats), the function write.rflp creates an output file containing the cluster group assignment for each sample. This text file can easily be processed further, for example, in spreadsheet software applications.

During a biodiversity study using PCR-RFLP, the resulting RFLP groups must be verified by sequencing two or more samples from each cluster and checking their identity, to ensure that the cutting height and classification of fragment patterns were low enough so that the aggregation of different genotypes in one cluster is avoided. To facilitate the rapid identification of PCR-RFLP samples, two or more representative samples (PCR products) from each detected similarity group can be sequenced, and the resulting nucleotide sequences compared with data in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) or other sequence databases. Reference data sets including band patterns and taxonomic affiliations might subsequently be established.

Common phylogenetic approaches require alignments of sequences. This is not a problem with highly similar sequences, but sequences deriving from environmental samples might include organisms of widely different taxa, which might be aligned with difficulty. We recommend an approach that includes pairwise similarities, calculated by the BLAST standalone tool, and enables the comparison of different taxa with no need for a detailed alignment. The success of this approach was shown in Peršoh *et al.* (2010), Peršoh & Rambold (2012), Flessa *et al.* (2012) and Flessa & Rambold (2013). RFLPtools enables the discrimination of DNA sequences via tabular report files of standalone BLAST (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release). Therefore, the similarity values for all-vs.-all BLAST results of DNA sequences generated with standalone BLAST from NCBI or local BLAST, as implemented in BioEdit v7.0.9 (Hall 1999), for example, are used to build a similarity matrix. The BLAST search tool is not implemented in RFLPtools, but tabular output files can easily be used for data exchange between the above-mentioned software programs. To import and analyse BLAST tabular report files, RFLPtools provides the function read.blast. Subsequently, the data frame obtained is modified into a similarity matrix. Details of the algorithm implemented to compute the similarity between samples can be looked up in the help files of the package. A visualization of the similarity matrix is possible using, for example, simPlot (R package MKmisc; Kohl 2012). As a terminal step, identified RFLP samples can be used to establish a reference data set. In the case of an existing reference data set containing RFLP fragment patterns and already identified taxon names (e.g. via DNA sequencing or BLAST search results), a comparison of RFLP genotypes derived from a new study with the existing reference samples is possible and enables the detection of known genotypes (function RFLPrefdist). The implemented method RFLPrefplot facilitates a visual comparison of new samples with reference samples (see Fig. 2). With these options, RFLPtools provides a PCR-RFLP-adapted application, which overcomes general problems when handling RFLP-derived data.

As we are generating objects of basic data types, the resulting compatibility with other R packages (i.e. population genetic packages) and analysis methods is a further advantage of RFLPtools. For example, it can be used with the popgen package, which uses R standard datatypes such as matrix, array and vector, or with the adegenet package, which contains several convert functions; that is, R standard datatypes can easily be converted to objects of adegenet classes.

Application example

To demonstrate the application advantages of RFLPtools, we selected a data set (see Appendix S1, Supporting information) including 112 RFLP types generated from double-stranded fungal ITS rRNA sequences obtained from cultivated fungi in previous studies (Triebel *et al.* 2005; Peršoh *et al.* 2010). Sequences were deposited at EMBL under the accession nos FR773168–FR773170, FR773172, FR773288, FR773289, FR773296, FR773297, FR773300, FR773304–FR773306, FR773308, FR773321, FR774049–FR774075, FR774077–FR774079, FR774081, FR774084, FR774089, FR774090, FR774092, FR774098, FR774101–FR774115, FR774117–FR774161.

Taxonomic names were assigned to the sequences using MegaBLAST (Zhang *et al.* 2000) at the NCBI website (http://www.ncbi.nlm.nih.gov; status: January 2010). A consensus name was compiled from the names under which sequences obtaining a 'bitscore' of at least 90% of the best matching sequences were deposited, following the approach of Peršoh *et al.* (2010). The nomenclature and classification concepts applied follow Index Fungorum (http://www.indexfungorum.org) and Myconet (http://www.fieldmuseum.org/myconet).

Restriction fragment length polymorphism band patterns were artificially created by cutting the ITS sequences with *Alu*I and *Msp*I in a virtual digest with the online application 'RestrictionMapper' (http://www.restrictionmapper.org/) and were stored in a text format suitable for the read.blast function, containing the sample name, number of bands and molecular weight of the band. The RFLP patterns of four sequences (FR773320, FR773324, FR774080 and 774082) were manually manipulated to

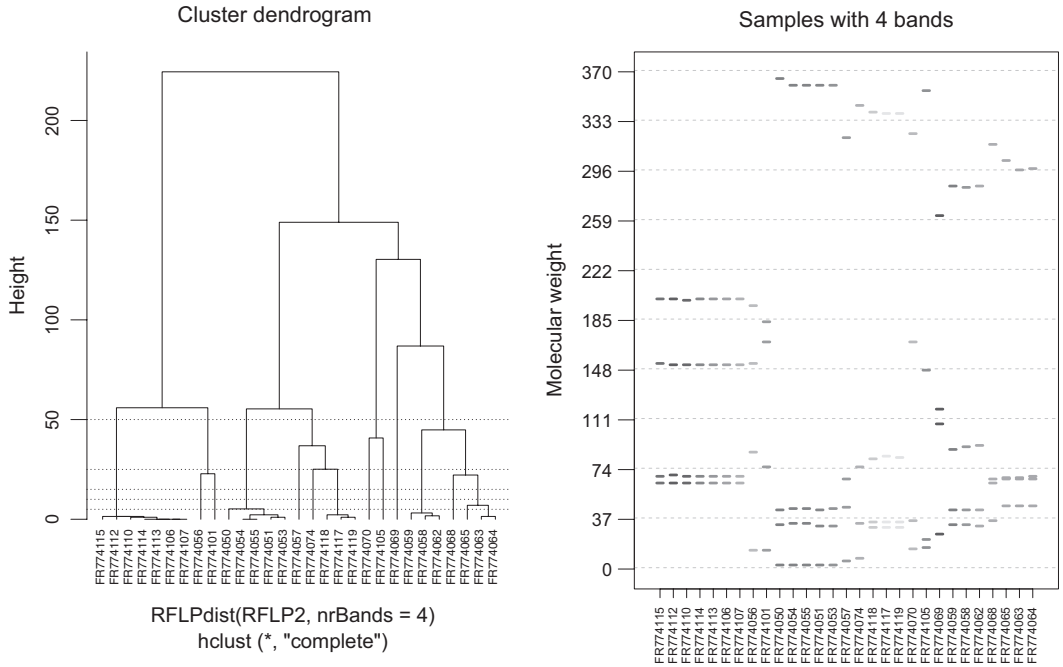

Fig. 2 Visual comparison of molecular weights from an example data set containing 29 RFLP samples exhibiting four fragments.

generate 'problematic' band patterns (in FR773320 and FR773324, the first band was deleted to simulate an overseen band, and in FR774070b and 774049b, which were manipulated duplicates from samples FR774070 and FR774049, one band was duplicated and modified with a difference in the molecular weight by three base pairs, to simulate an overexpressed thick band, which was identified as two bands by gel analysis software).

Subsequently, the RFLP samples were grouped by fragment numbers (function nrBands), resulting in eight groups containing RFLP samples with 1–8 bands, respectively. By applying the quality check function (RFLPqc, QC.lo = 0.9, QC.up = 1.1) on the data set, the four manipulated RFLP samples fell outside the range (the sum of bands of sample FR773320 was out of range by 40.57% and that of FR773324 by 40.58%, FR774080 by 131.07% and FR774082 by 127.29%) and were excluded from further analysis.

Distances between fragment patterns of RFLP samples within each of these groups were computed with the function RFLPdist. Subsequently, hierarchical cluster analysis of RFLP samples was performed using the function hclust (R base package stats). To extract groups of identical samples, the resultant dendrograms were cut (cutree, R base package stats) at heights of 5, 10, 15 and 25 to compare the resulting RFLP clusters and identify the optimal cutting height for these data. In our example, the resulting RFLP cluster can easily be compared with the genotype affiliation of each sample (for results, see Appendix S2, Supporting information). Therefore, the similarity of samples was plotted using dendrograms and parallel molecular weight patterns (function RFLPplot) (see Fig. 2). After cutting the dendrograms, the function write.hclust was applied to write an output file containing the cluster group assignment for each sample [included in Appendix S2, Supporting information (cluster number)].

This procedure results in 51 different RFLP groups at a height of 5. This height appears optimal for the data set used, because there is no RFLP cluster with different fungal genotypes at a height of $h = 5$. However, some of the 33 fungal genotypes are split into different RFLP clusters [*Aureobasidium pullulans*-1 (3 cluster), *Botryosphaeria*-1 (2 cluster), *Capnocheirides*-1 (2 cluster), *Cladosporium*-1 (3 cluster), *Cladosporium*-2 (5 cluster), *Phialocephala*-1 (2 cluster), *Fusarium*-1 (2 cluster), *Lewia*-1 (2 cluster), *and Vibrisseaceae*-1 (2 cluster)], but all of these clusters are homogeneous. At a height of $h = 10$, one cluster is nonhomogeneous (*Cladosporium*-1 and *Cladosporium*-2 are in the same group). In this data set, with $h = 5$, no fungal genotype was overseen, but there is a requirement for sequence identification of each RFLP cluster to detect genotypes divided into different RFLP types, due to mutations in the sequence. Identification of split groups is important to avoid an overestimation of the fungal diversity. If RFLPtools is used as a presort tool in diversity studies, there is no need to sequence each individual RFLP sample.

Conclusion

Polymerase chain reaction-restriction fragment length polymorphism is a common and applicable method in

diversity studies, but a suitable, freely available analysis application that provides interoperability, flexibility and compatibility with further statistical analysis methods is still needed. Although several software applications exist for PCR-RFLP fragment pattern analysis, RFLPtools represents the first purpose-built application for PCR-RFLP in R. R packages have the advantage that statistical analyses are easily applicable to the resulting outputs, and as objects of basic data types are generated, the resulting compatibility with other R packages and analysis methods are a further advantage of RFLPtools.

Because its function composition follows the workflow of PCR-RFLP analysis, RFLPtools allows reliable data processing from the fragment band pattern to the identification of RFLP samples. Furthermore, in contrast to existing applications, RFLPtools provides a quality check for the input data. This option for data quality checking and band pattern visualization supports the handling of problematic data by detecting invalid samples and allows the subsequent rechecking or exclusion of those samples, which improves data quality. As RFLPtools was designed for data gathered from images of electrophoresis gels, the package contains some functions to handle biased data. However, the additional functions might be applied to data gathered from capillary sequencers using fluorescently labelled primers.

The main advantage of RFLPtools is that it uses distances of the molecular weights of fragments instead of band matching. Options to generate a reference data set of identified RFLP samples based on DNA sequences and to report files derived from all-vs.-all BLAST searches allow for the comparison of new samples with known genotypes, supplementing the application. This is completed by a second option for visualization of band patterns of new samples and references. With these options, RFLPtools provides a PCR-RFLP-adapted application which considers general problems encountered when handling RFLP-derived data.

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A.K. and F.F. designed the research; M.K. programmed and described the functions in the manuscript, F.F. performed the analysis and wrote the description of the application example, A.K. and F.F. wrote the paper.

Data Accessibility

The software package is freely available from 'The Comprehensive R Archive Network' (CRAN) at http://cran.r-project.org/web/packages/RFLPtools.

DNA sequences: GenBank accessions FR773168–FR773170, FR773172, FR773288, FR773289, FR773296,

FR773297, FR773300, FR773304–FR773306, FR773308, FR773321, FR774049–FR774075, FR774077–FR774079, FR774081, FR774084, FR774089, FR774090, FR774092, FR774098, FR774101–FR774115, FR774117–FR774161.

Supporting Information

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

Appendix S1 Example dataset.

Appendix S2 Comparison of four different cutting heights of resultant dendrograms by applying cutree on the example dataset (section application example).

Electronic appendix 2

Comparison of four different cutting heights of resultant dendograms by applying cutree on dendrograms which were obtained by applying the function hclust on a dataset comprising 110 samples which were previously assigned to 33 genotypes. Cluster names include the number of bands (Bd) and cluster number on h = 5, h = 10, h = 15, h = 25, and h = 50. There is no RFLP-cluster including two or more fungal genotypes at a height of 5.

Sample-ID	Sample Name assignment	Cluster ID H5	Cluster ID H10	Cluster ID H15	Cluster ID H25	Cluster ID H50
FR774049	*Truncatella betulae*-1	Bd2_H5_1	Bd2_H10_1	Bd2_H15_1	Bd2_H25_1	Bd2_H50_1
FR774077	*Coprinus*-1	Bd2_H5_2	Bd2_H10_2	Bd2_H15_2	Bd2_H25_2	Bd2_H50_2
FR774061	*Gibberella*-1	Bd3_H5_1	Bd3_H10_1	Bd3_H15_1	Bd3_H25_1	Bd3_H50_1
FR774081	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774084	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774089	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774090	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774092	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774098	*Phoma*-1	Bd3_H5_2	Bd3_H10_2	Bd3_H15_2	Bd3_H25_2	Bd3_H50_2
FR774108	*Aureobasidium pullulans*-1	Bd3_H5_3	Bd3_H10_3	Bd3_H15_3	Bd3_H25_3	Bd3_H50_3
FR774109	*Aureobasidium pullulans*-1	Bd3_H5_3	Bd3_H10_3	Bd3_H15_3	Bd3_H25_3	Bd3_H50_3
FR774160	*Sphaeropsis sapinea*-1	Bd3_H5_4	Bd3_H10_4	Bd3_H15_4	Bd3_H25_4	Bd3_H50_4
FR774050	*Phialocephala*-1	Bd4_H5_01	Bd4_H10_01	Bd4_H15_01	Bd4_H25_01	Bd4_H50_1
FR774051	*Phialocephala*-1	Bd4_H5_02	Bd4_H10_01	Bd4_H15_01	Bd4_H25_01	Bd4_H50_1
FR774053	*Phialocephala*-1	Bd4_H5_02	Bd4_H10_01	Bd4_H15_01	Bd4_H25_01	Bd4_H50_1
FR774054	*Phialocephala*-1	Bd4_H5_02	Bd4_H10_01	Bd4_H15_01	Bd4_H25_01	Bd4_H50_1
FR774055	*Phialocephala*-1	Bd4_H5_02	Bd4_H10_01	Bd4_H15_01	Bd4_H25_01	Bd4_H50_1
FR774056	Helotiales-1	Bd4_H5_03	Bd4_H10_02	Bd4_H15_02	Bd4_H25_02	Bd4_H50_2
FR774057	*Preussia dubia*-1	Bd4_H5_04	Bd4_H10_03	Bd4_H15_03	Bd4_H25_03	Bd4_H50_3
FR774058	*Fusarium*-1	Bd4_H5_05	Bd4_H10_04	Bd4_H15_04	Bd4_H25_04	Bd4_H50_4
FR774059	*Fusarium*-1	Bd4_H5_05	Bd4_H10_04	Bd4_H15_04	Bd4_H25_04	Bd4_H50_4
FR774062	*Fusarium*-1	Bd4_H5_05	Bd4_H10_04	Bd4_H15_04	Bd4_H25_04	Bd4_H50_4
FR774063	*Lewia*-1	Bd4_H5_06	Bd4_H10_05	Bd4_H15_05	Bd4_H25_05	Bd4_H50_4
FR774064	*Lewia*-1	Bd4_H5_06	Bd4_H10_05	Bd4_H15_05	Bd4_H25_05	Bd4_H50_4
FR774065	*Lewia*-1	Bd4_H5_07	Bd4_H10_05	Bd4_H15_05	Bd4_H25_05	Bd4_H50_4
FR774068	*Monodictys arctica*-1	Bd4_H5_08	Bd4_H10_06	Bd4_H15_06	Bd4_H25_05	Bd4_H50_4
FR774069	*Cryptococcus*-1	Bd4_H5_09	Bd4_H10_07	Bd4_H15_07	Bd4_H25_06	Bd4_H50_5
FR774070	*Camarosporium*-1	Bd4_H5_10	Bd4_H10_08	Bd4_H15_08	Bd4_H25_07	Bd4_H50_6
FR774074	Ascomycota-1	Bd4_H5_11	Bd4_H10_09	Bd4_H15_09	Bd4_H25_08	Bd4_H50_3
FR774101	*Ascocalyx abietina*-1	Bd4_H5_12	Bd4_H10_10	Bd4_H15_10	Bd4_H25_02	Bd4_H50_2
FR774105	*Cryptococcus wieringae*-1	Bd4_H5_13	Bd4_H10_11	Bd4_H15_11	Bd4_H25_09	Bd4_H50_6
FR774106	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774107	*Aureobasidium*	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7

	pullulans-1					
FR774110	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774112	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774113	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774114	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774115	*Aureobasidium pullulans*-1	Bd4_H5_14	Bd4_H10_12	Bd4_H15_12	Bd4_H25_10	Bd4_H50_7
FR774117	*Alternaria*-1	Bd4_H5_15	Bd4_H10_13	Bd4_H15_13	Bd4_H25_11	Bd4_H50_3
FR774118	*Alternaria*-1	Bd4_H5_15	Bd4_H10_13	Bd4_H15_13	Bd4_H25_11	Bd4_H50_3
FR774119	*Alternaria*-1	Bd4_H5_15	Bd4_H10_13	Bd4_H15_13	Bd4_H25_11	Bd4_H50_3
FR773168	Vibrisseaceae-1	Bd5_H5_01	Bd5_H10_01	Bd5_H15_01	Bd5_H25_01	Bd5_H50_1
FR773169	Vibrisseaceae-1	Bd5_H5_02	Bd5_H10_01	Bd5_H15_01	Bd5_H25_01	Bd5_H50_1
FR773170	Vibrisseaceae-1	Bd5_H5_01	Bd5_H10_01	Bd5_H15_01	Bd5_H25_01	Bd5_H50_1
FR773172	Vibrisseaceae-1	Bd5_H5_01	Bd5_H10_01	Bd5_H15_01	Bd5_H25_01	Bd5_H50_1
FR773288	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773289	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773296	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773297	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773300	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773304	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773305	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773306	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773308	Capnocheirides-1	Bd5_H5_03	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR773321	Capnocheirides-1	Bd5_H5_04	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR774072	Capnocheirides-1	Bd5_H5_09	Bd5_H10_02	Bd5_H15_02	Bd5_H25_02	Bd5_H50_2
FR774052	*Phialocephala*-1	Bd5_H5_05	Bd5_H10_03	Bd5_H15_03	Bd5_H25_03	Bd5_H50_3
FR774060	*Fusarium*-1	Bd5_H5_06	Bd5_H10_04	Bd5_H15_04	Bd5_H25_04	Bd5_H50_1
FR774066	*Neurospora*-1	Bd5_H5_07	Bd5_H10_05	Bd5_H15_05	Bd5_H25_05	Bd5_H50_4
FR774067	*Neurospora*-1	Bd5_H5_07	Bd5_H10_05	Bd5_H15_05	Bd5_H25_05	Bd5_H50_4
FR774071	*Leucosporidium*-1	Bd5_H5_08	Bd5_H10_06	Bd5_H15_06	Bd5_H25_06	Bd5_H50_5
FR774073	Mycota-2	Bd5_H5_10	Bd5_H10_07	Bd5_H15_07	Bd5_H25_07	Bd5_H50_6
FR774075	Ascomycete-1	Bd5_H5_11	Bd5_H10_08	Bd5_H15_08	Bd5_H25_08	Bd5_H50_7
FR774079	*Phacidiopycnis*-1	Bd5_H5_12	Bd5_H10_09	Bd5_H15_09	Bd5_H25_09	Bd5_H50_6
FR774102	*Physalospora vaccinii*-1	Bd5_H5_13	Bd5_H10_10	Bd5_H15_10	Bd5_H25_10	Bd5_H50_8
FR774104	*Preussia*-1	Bd5_H5_14	Bd5_H10_11	Bd5_H15_11	Bd5_H25_11	Bd5_H50_1
FR774111	*Aureobasidium pullulans*-1	Bd5_H5_15	Bd5_H10_12	Bd5_H15_12	Bd5_H25_12	Bd5_H50_4
FR774120	*Botryosphaeria*-1	Bd5_H5_16	Bd5_H10_13	Bd5_H15_13	Bd5_H25_13	Bd5_H50_9
FR774145	*Cladosporium*-2	Bd5_H5_17	Bd5_H10_14	Bd5_H15_14	Bd5_H25_14	Bd5_H50_8
FR774121	*Botryosphaeria*-1	Bd6_H5_1	Bd6_H10_1	Bd6_H15_1	Bd6_H25_1	Bd6_H50_1
FR774122	*Botryosphaeria*-1	Bd6_H5_1	Bd6_H10_1	Bd6_H15_1	Bd6_H25_1	Bd6_H50_1
FR774131	*Cladosporium*-1	Bd6_H5_2	Bd6_H10_2	Bd6_H15_2	Bd6_H25_2	Bd6_H50_2
FR774150	*Cladosporium*-1	Bd6_H5_2	Bd6_H10_2	Bd6_H15_2	Bd6_H25_2	Bd6_H50_2
FR774151	*Cladosporium*-1	Bd6_H5_2	Bd6_H10_2	Bd6_H15_2	Bd6_H25_2	Bd6_H50_2
FR774126	*Cladosporium*-1	Bd6_H5_2	Bd6_H10_2	Bd6_H15_2	Bd6_H25_2	Bd6_H50_2
FR774130	*Cladosporium*-1	Bd6_H5_2	Bd6_H10_2	Bd6_H15_2	Bd6_H25_2	Bd6_H50_2
FR774147	*Cladosporium*-2	Bd6_H5_3	Bd6_H10_3	Bd6_H15_3	Bd6_H25_2	Bd6_H50_2
FR774078	Mycota-3	Bd7_H5_1	Bd7_H10_1	Bd7_H15_1	Bd7_H25_1	Bd7_H50_1
FR774103	*Cryptosporiopsis*-1	Bd7_H5_2	Bd7_H10_2	Bd7_H15_2	Bd7_H25_2	Bd7_H50_2
FR774123	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2

FR774125	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774132	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774133	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774134	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774135	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774136	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774137	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774138	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774139	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774140	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774141	*Cladosporium*-1	Bd7_H5_3	Bd7_H10_3	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774124	*Cladosporium*-1	Bd7_H5_4	Bd7_H10_4	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774128	*Cladosporium*-2	Bd7_H5_5	Bd7_H10_4	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774129	*Cladosporium*-2	Bd7_H5_5	Bd7_H10_4	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774148	*Cladosporium*-2	Bd7_H5_5	Bd7_H10_4	Bd7_H15_3	Bd7_H25_3	Bd7_H50_2
FR774143	*Cladosporium*-2	Bd7_H5_6	Bd7_H10_5	Bd7_H15_4	Bd7_H25_3	Bd7_H50_2
FR774153	*Penicillium*-2	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774154	*Penicillium*-2	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774155	*Penicillium*-2	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774156	*Penicillium*-2	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774157	*Penicillium-2*	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774158	*Penicillium-2*	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774159	*Penicillium-2*	Bd7_H5_7	Bd7_H10_6	Bd7_H15_5	Bd7_H25_4	Bd7_H50_3
FR774161	Mycota-1	Bd7_H5_8	Bd7_H10_7	Bd7_H15_6	Bd7_H25_5	Bd7_H50_2
FR774127	*Cladosporium*-2	Bd8_H5_1	Bd8_H10_1	Bd8_H15_1	Bd8_H25_1	Bd8_H50_1
FR774142	*Cladosporium*-2	Bd8_H5_1	Bd8_H10_1	Bd8_H15_1	Bd8_H25_1	Bd8_H50_1
FR774144	*Cladosporium*-2	Bd8_H5_1	Bd8_H10_1	Bd8_H15_1	Bd8_H25_1	Bd8_H50_1
FR774146	*Cladosporium*-2	Bd8_H5_1	Bd8_H10_1	Bd8_H15_1	Bd8_H25_1	Bd8_H50_1
FR774149	*Cladosporium*-2	Bd8_H5_1	Bd8_H10_1	Bd8_H15_1	Bd8_H25_1	Bd8_H50_1
FR774152	*Penicillium*-1	Bd8_H5_2	Bd8_H10_2	Bd8_H15_2	Bd8_H25_2	Bd8_H50_2
total number of RFLPcluster		**51**	**45**	**44**	**41**	**39**

13 List of all own publications

Core articles of this PhD project:

1. **Flessa F.**, Peršoh D, Rambold G. (2012): Annuality of Central European deciduous tree leaves delimits community development of epifoliar pigmented fungi. – Fungal Ecology **5** (5): 554-561.
2. **Flessa F.** and Rambold G. (2013): Diversity of the *Capnocheirides rhododendri*-dominated fungal community in the phyllosphere of *Rhododendron ferrugineum* L. – Nova Hedwigia **97**: 19-53.
3. **Flessa F.**, Harjes J., Cáceres M. S., Rambold G.: Comparative analyses of sooty mould communities from Brazil and Central Europe. Mycological Progress (published online, 2021).
4. **Flessa F.**, Kehl A., W. Babel, G. Rambold, M. Kohl: Effects of sap-feeding insects, plant characteristics and weather parameters on sooty moulds in the temperate zone. Manuscript in preparation for submission to Oecologia.

Methodical articles in the context of the Ph.D. project:

5. Pietrowski A., **Flessa F.**, Rambold G. (2012): Towards an efficient phenotypic classification of fungal cultures from environmental samples using digital imagery. – Mycological Progress 11 (2): 383-393.
6. **Flessa F.**, Kehl A., Kohl M. (2013): Analysing diversity and community structures using PCR-RFLP: a new software application. – Molecular Ecology Resources **13**: 726-733.

Further publications not included in this PhD project:

7. Peršoh D., Melcher M., **Flessa F.**, Rambold G. (2010): First fungal community analyses of endophytic ascomycetes associated with *Viscum album* ssp. *austriacum* and its host *Pinus sylvestris*. – Fungal Biology **114:** 585-596.
8. Stadler M., Fournier J., Læssøe T., Chlebicki A., Lechat C., **Flessa F.**, Rambold G., Peršoh D. (2010): Chemotaxonomic and phylogenetic studies of *Thamnomyces* (Xylariaceae) – Mycoscience **51** (3) 189-207.
9. Fournier J., **Flessa F.**, Peršoh D, Stadler M. (2011): Three new *Xylaria* species from southwestern Europe. – Mycological Progress **10** (1) 33-52.

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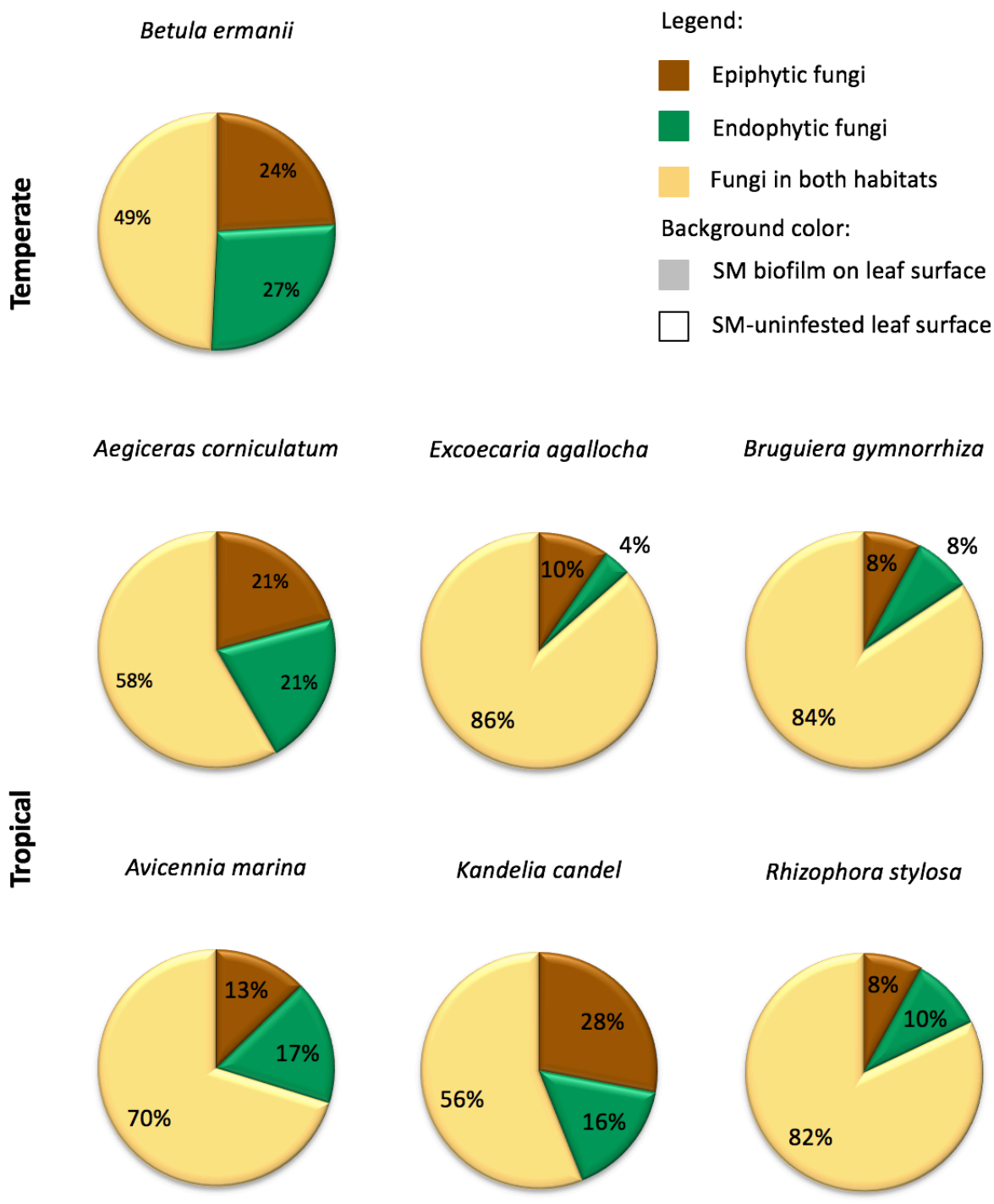
15 Appendix



Appendix Figure 1 Relative amounts of exclusively epiphytic fungi, exclusively endophytic, and fungi common in both communities in the phyllosphere of leaves from six different host plants from a tropical mangrove ecosystem and one host plant from a temperate region. Results were obtained by MB approaches.

Appendix Table 1 Additional sequences of fungi from SM mycobiomes on twigs of *R. ferrugineum*, which were not included in the manuscripts of this study. All sequences were deposited and published in GenBank.

GenBank Accession number	**Assigned name**
FR773242	Ascomycota-3
FR773241	*Aureobasidium pullulans*-1
FR773253	*Aureobasidium pullulans*-1
FR773404	*Capnocheirides rhododendri*-1
FR773475	*Capnocheirides rhododendri*-1
FR773235	*Cladosporium*-3
FR773238	*Cladosporium*-3
FR773243	*Cladosporium*-3
FR773262	*Cladosporium*-3
FR773342	*Cladosporium*-3
FR773256	*Cladosporium*-4
FR773254	*Dothichiza pityophila*-1
FR773471	*Dothichiza pityophila*-1
FR773354	Hyaloscyphaceae-1
FR773353	Leotiomycetidae-2
FR773355	Mycota-1
FR773233	*Phaeosphaeria*-1
FR773400	*Phoma macrostoma*-1
FR773352	*Phoma*-1
FR773403	*Phoma*-1
FR773401	*Sydowia*-2

16 (Eidesstattliche) Versicherungen und Erklärungen

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbstständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6 BayHSchG).

(§ 8 Satz 2 Nr. 3 PromO Fakultät)

Hiermit erkläre ich, dass ich die Dissertation nicht bereits zur Erlangung eines akademischen Grades eingereicht habe und dass ich nicht bereits diese oder eine gleichartige Doktorprüfung endgültig nicht bestanden habe.

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Ipsach, 12.02.2022, Fabienne Flessa