

Fungi associated with woody tissues of *Acer pseudoplatanus* in forest stands with different health status concerning sooty bark disease (*Cryptostroma corticale*)

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Abstract

In previous years, namely 2018–2020, Germany experienced periods of exceptional weather conditions. Extremely high summer temperatures and precipitation deficits induced stress and mortality in forest trees. *Acer pseudoplatanus* (sycamore) was one of the affected tree species. Symptoms of the Sooty Bark Disease (SBD) and severe damage of entire stands, both caused by the invasive fungal species *Cryptostroma corticale*, were reported more frequently. In order to get a better understanding of the distribution of *C. corticale*, case reports of SBD for Germany, in general, as well as for the federal state of Hesse, in particular, are compiled and presented. To explore the endophytic distribution of *C. corticale*, wood cores from visibly healthy sycamore stems were sampled and all outgrowing fungi were determined and recorded. In total, 50 trees, aged 30–65 years, were sampled at five different forest stands and from which 91 endophytic filamentous fungal species could be isolated. The fungal endophytic community in the woody tissue of the sycamore trees varied greatly at the different sites and between the trees. The number of endophytic species isolated at the different sites ranged from 13 to 43 and no species was found at all sites. At 1.20 m stem height, 3.3 endophyte species could be isolated from woody tissue per tree on average. The most abundant species isolated from visibly healthy sycamore in regard to both occurrence at the studied sites and continuity was *C. corticale*. It was recorded at four studied forest stands, from 26% of all studied sycamore trees and had a frequency of 7.85% relative to the 293 isolated filamentous strains that were isolated. The second most abundant species was *Xylaria longipes* followed by *Lopadostoma turgidum*.

1. Introduction

Sycamore (*Acer pseudoplatanus* L., *Sapindaceae*) is a deciduous tree that can be found throughout large parts of Europe. Its most northern distribution reaches into Lithuania, while the southern distribution reaches the south of Italy (EUFORGEN 2022). In Germany, besides *A. pseudoplatanus*, two other native *Acer* species occur, namely *Acer platanoides* L. and *Acer campestre* L. Pure maple stands, if found in Germany, are the result of active management, since no natural pure *Acer* stands exist in this region. According to the phytosociological assignment and syntaxonomical treatment following Runge (1994), maple occurs in subalpine sycamore-beech forests (*Aceri-Fagion*) or in lime-maple mixed forests (*Tilio-Acerion*).

In German forestry, maple is often found in mixed forests, on calcareous soil with good nutrient and good water supply. The timber is very durable and thus often used for furniture and floors. Additionally, the occasionally wavy grained wood is used for the manufacture of musical instruments, making sycamore a valuable timber species (EUFORGEN 2022). Sycamore is a very valuable urban tree species as well. In Germany, 7.1% of the forested tree species are assigned to a group called “broad leaved trees with high life expectancy”, which includes *Acer* sp., *Platanus* sp., *Fraxinus excelsior* L., *Tilia* sp., *Robinia pseudoacacia* L. and *Ulmus* sp. The forest communities in which *A. pseudoplatanus* can be found are stands of the *Fagion sylvaticae* plant community, including mixed stands of beech and highly valuable hardwoods. It can also be found in other mixed stands of highly valuable hardwoods (*Tilio-Acerion*) and stands that are strongly influenced by water (*Alno-Ulmion* and *Carpinion*), and less frequently mixed coniferous stands (*Piceion*; Schmidt 2009).

Even though many fungi are reported from *A. pseudoplatanus* (244 different species according to the USDA website, Farr and Rossman 2022), only a few studies focus specifically on endophytes of sycamore or fungi associated with the living woody tissue of sycamore (Kowalski and Kehr 1992; Kelnarová et al. 2017). The available data on fungi associated with *A. pseudoplatanus* mainly refers to dead wood (Butin and Kowalski 1986; Chlebicki 1988; Unterseher et al. 2005; Brglez et al. 2020a) or to leaves (Schlegel et al. 2018).

According to Petrini (1991), Saikkonen et al. (1998), Arnold and Lutzoni (2007), and Sieber (2007), we consider those fungi as endophytes that spend a significant amount of their life cycle within the host plant tissue without causing symptoms. A change of environmental conditions can cause a change in the life style of the fungus from endophytic to pathogenic (Sieber 2007) or to saprotrophic when the host tissue dies (Sun et al. 2011). Some fungi can be endophytic in one tree species and pathogenic in another tree species (Petrini 1991; Saikkonen et al. 2010; Sanz-Ros et al. 2015). In other cases, endophytes can change to a pathogenic lifestyle under the right circumstances, such as stress in the host tree and high summer temperatures (Ragazzi et al. 2003; Hyde and Soytong 2008). Many wood-decay fungi appear to have a transient endophytic lifestyle, which may be in preparation for their saprotrophic life stage (Boddy and Rayner 1983; Parfitt et al. 2010).

As a result of the very warm and dry summers and mild winters in the years 2018–2020, many forest trees started showing signs of stress and mortality. In Germany these years were characterised by temperatures averaging from 19.3°C during the summer months in

2018 (3°C above average of the reference period from 1981–2010), to 19.2°C in 2019 (2.9°C above average of the reference period) and 18.2°C in 2020 (1.9°C above average of the reference period), (DWD 2018, 2019, 2020). The precipitation for the three summer months was 130 l/m² in 2018, 175 l/m² in 2019 and 230 l/m² in 2020, while the required average precipitation for the reference period was 239 l/m² (DWD 2018, 2019, 2020). As a consequence, German forests developed a soil water deficit resulting in signs of stress in the trees (NW-FVA 2020, 2021). One of these tree species was sycamore, and following these weather extremes an extraordinary outbreak of Sooty Bark Disease (SBD) was observed (Bork 2018; Delb et al. 2019; Rohde et al. 2019; Wenzel et al. 2019).

Cryptostroma corticale (Ellis & Everh.) P.H. Greg. & S. Waller, the causal agent of SBD (Ellis and Everhart, 1889; Gregory and Waller 1951), was observed more frequently, starting 2018, in several regions in Germany causing obvious symptoms on trees. Symptoms included wilting and dieback in the crown in earlier stages, as well as the production of masses of black conidia under the outer layer of the bark in later stages of the disease (Enderle et al. 2020; Schlößer and Langer 2021). *Cryptostroma corticale* is presumed to be opportunistic with endophytic, pathogenic and saprophytic life stages that react to stress, and it is known to have an optimal growing temperature of 25°C (Dickenson 1980). It is assumed that *C. corticale* primarily infects the tree through fresh wounds (Townrow 1953; Dickenson 1980) and causes a soft rot in infested tissues of the tree, like many other closely related fungi from the *Xylariales* (Worrall et al. 1997; Schwarze 2018). The original description of *C. corticale* by Ellis and Everhart (1889) originated from Canada. The first record in Germany reported by the plant protection office in Berlin dates back to 1964 (Plate and Schneider 1965). In this case, spores of *C. corticale* were detected on infested firewood originating from trees of the Berlin Tiergarten park area and stored in a basement. Triggered by extremely warm weather and precipitation deficits in the year 2003, several incidences of SBD occurred in this and the following years throughout Germany and Europe (Cech 2004; Engesser et al. 2004; Metzler 2006; Robeck et al. 2008; Langer et al. 2013; Bencheva 2014; Koukol et al. 2014). The recently observed cases of disease from 2018 to 2021 constitute the biggest outbreaks of SBD in Germany yet.

The goal of this research was to undertake a better assessment of the current status and distribution of *C. corticale* as well as the potential risks of SBD in Germany. Therefore the distribution of SBD in German forests was mapped and fungal endophytes of sycamore woody stem tissue were studied. In order to explore the endophytes of sycamore, as well as the endophytic (non-symptomatic) distribution of *C. corticale* in visibly healthy trees, a study was conducted examining stem wood cores, following an adjusted version of the method used by Kelnarová et al. (2017). Different study sites were chosen throughout the federal state of Hesse, in which a high precipitation deficit was recorded for 2018, 2019 and 2020, as well as one forest plot in Schleswig-Holstein, where no or only a minor deficit in precipitation was recorded in those years.

2. Materials And Methods

2.1 Mapping of the Sooty Bark Disease

In order to map the distribution of SBD cases in Germany, infestation data from the federal forest protection institutions of Germany were compiled using Universal Transverse Mercator (Grid = UTM; deadline 31.05.2021). Additionally, from May 2020 to September 2020, 123 forest stands with *A. pseudoplatanus* (aged 20–100 years, with sycamore as a dominant tree species) in Hesse were evaluated with regard to visible symptoms of sooty bark disease. In addition, data on cases of SBD in Schleswig-Holstein, Lower Saxony, Hesse, and Saxony-Anhalt were retrieved from the 'Waldschutzmeldeportal' Forest Protection Reporting Portal (WSMP) of the Northwest German Forest Research Institute (NW-FVA) or directly reported by forest owners or foresters and checked by the authors between May 2020 and May 2021. Forest owners and foresters can register georeferenced forest damage in the online WSMP. QGIS (v. 3.22.3) (www.qgis.org) was used to create a combined map based on a preliminary distribution map published by Schlößer and Langer (2021).

2.2 Sampling sites

Wood samples were taken from five forest stands (Table 1) containing sycamore, located in two federal states of Germany. Four of the sites were located in Hesse in the middle of Germany and one, Nehnten (date of sampling: 30. – 31.09.2021), in Schleswig-Holstein, in the very north of Germany. The four Hessian sites were located in the forest departments of Melsungen (date of sampling: 10.03.2021), Nidda (date of sampling: 13.07.2021), Fulda (date of sampling: 04.05.2021) and Beerfelden (date of sampling: 09. – 10.09.2021). Two of the five stands, in Fulda and in Nidda, were visibly affected by SDB at the time of sampling, and several maple trees exhibited black conidia underneath the ruptured bark. In the studied stands in Melsungen, Beerfelden and Nehnten, no visible signs of SBD were observed. The forest stands without symptoms of SBD were located at different distances from the nearest known

infestation point with *C. corticale*. The studied site in Beerfelden was 200 m away from the next SBD affected trees, while in Melsungen there was more than 30 km of aerial distance to the closest infested stand. In Schleswig-Holstein, no case of SBD in forests had been reported until June 2022.

Table 1
Sampling site conditions; apart from Nehmten (mesotrophic) all other studied forest sites are eutrophic. UTM = Universal Transverse Mercator (Grid).

Forest site (Coordinates UTM)	Elevation above sea level (m)	Exposition	Inclination	Climate	Soil water supply	Bedrock	stand tree components	Visible signs of SBD	<i>C. corticale</i> isolated
Beerfelden (32 U 485720 5492792)	450–500	north and south	moderately inclined (0°-18°)	strongly subatlantic	slightly moist/ moist	granite with loamy loess	maple, sycamore, European beech	no	yes
Fulda (32 U 546727 5586419)	410–450	west	moderately inclined (0°-18°)	weakly subatlantic	slightly moist/ moist	basalt with loamy loess	hornbeam, European beech, maple	yes	no
Melsungen (32 U 539938 5674066)	470–490	hilltop	slightly inclined (0°-9°)	moderately subatlantic	moist/ changing water content	basalt with loamy loess	maple, European beech, European ash	no	yes
Nehmten (32 U 589629 5993467)	66	south- west	moderate inclination (0°-9°)	subatlantic	moist/ high water storing capacity	diluvial sand. boulder clay	sycamore	no	yes
Nidda (32 U 502557 5581999)	290–330	north	moderate to slight inclination (0°-18°)	weakly subatlantic	slightly dry/ slightly moist	basalt	European ash, sycamore	yes	yes

2.3 Isolation of fungi

At each site, ten obviously healthy trees, aged 30–65 years and without visible symptoms of SBD, were sampled using increment borers (Haglöf Increment Borer Mora-Coretax, three-edged, 300 mm drilling depth, 5.15 mm diameter). Two 20 cm increments of stem tissue per tree were taken at 1.20 m stem height above ground following the method of Kelnarová et al. (2017). In Melsungen, the first sampled site, three increments were taken per tree. The bark of the sample trees was sprayed with 70% Ethanol at sampling height and wiped with a paper towel. Two borers were used alternately and sterilised by flame shortly before every use. The outer layer of the bark was carefully scraped and the exposed tissue was disinfected with Ethanol again. Increments, taken on the vertical axis, were carefully removed from the extractor by disinfected hands and placed into clean and marked plastic tubes. The borer, extractor and knife were cleaned each time before taking a new sample by spraying with ethanol and wiping away the excess plant material, which was followed by flame sterilisation. Samples were cooled during transportation to the laboratory. Sampling was performed from March to September 2021. For each tree diameter at breast height (1.30 m), the following were recorded: crown vitality, the number of epicormic shoots as an indicator for vitality as well as visible signs of infestation with *C. corticale* or *Stegonsporium pyriforme* (Hoffm.) Corda (Online Resource 1).

In the laboratory, each increment was surface sterilised by rinsing the sample with 70% Ethanol and left to dry for a few minutes. Increments were cut into 5 mm long segments and three pieces were placed on each 90 mm petri dish containing malt yeast peptone (MYP) agar, modified according to Langer (1994) containing 0.7% malt extract (Merck 1.05391.0500, Darmstadt, Germany), 0.05% yeast extract (Fluka 70,161-100G, Seelze, Germany), 0.1% peptone (Merck 1.07272.0500), and 1.5% agar (Fluka 05040-1KG). From the sampling sites in Fulda, Beerfelden, Nidda and Nehmten, the increment segments with visible wood discoloration or signs of rot were observed and counted. The petri dishes were incubated at room temperature and with ambient daylight for four weeks. The cultures were monitored every second day in the first two weeks and twice a week in the third and fourth week. Emerging mycelia were sub-

cultured into pure cultures. The pure cultures were tentatively grouped into morphotypes (MTs). At least one representative culture for each MT was stored on MYP slants at 4°C in the fungal culture collection of the NW-FVA.

Frequency of isolated taxa, defined as proportion of isolated strains in relation to the total number of isolated filamentous strains, was calculated. Continuity of isolated taxa, defined as the number of trees from which the fungus was isolated in relation to the total number of trees, was calculated.

2.4 Molecular analysis

As a rule, one representative strain per MT was used for genetic analysis. Genomic DNA of the fungal cultures was extracted according to Izumitsu et al. (2012) with minor modifications. In addition, the fungal material was crushed before microwaving the sample. Mycelium was placed in 1.5 ml Eppendorf tubes (tubes) with five glass beads (3 mm) and 150 µl of TE buffer (10 ml 1 mmol Tris HCl (pH 0.8), 2 ml 0.5 mmol EDTA). The mycelium was crushed in a Retsch Mixer Mill MM200 (Retsch, Haan, Germany) and set at 25 vibrations per second for 90 seconds.

For all strains the 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers ITS-1 and ITS-2 (ITS) was amplified using the primer pair ITS-1F (Gardes and Bruns 1993) + ITS-4 (White et al. 1990). For strains belonging to *Neonectria*, the actin gene (ACT) and portion of the β -tubulin gene (TUB) were additionally amplified using primer pairs *Tact1* + *Tact2* (Samuels et al. 2006) and T1 (O'Donnell and Cigelnik 1997) + Bt-2b (Glass and Donaldson 1995), respectively. The PCR mixture consisted of 1 µl of DNA and 19 µl master mix which contained 2.5 µl 10x buffer (Carl Roth, Karlsruhe, Germany), 1 µl of each primer (10 mmol, 2.5 µl MgCl₂ (25 mmol), 0.1 µl Taq polymerase (Carl Roth, Karlsruhe, Germany) and 2.5 µl of 2 mmol dNTPs (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Each reaction was topped up to a volume of 20 µl by adding sterile water.

A StepOnePlus™ PCR System (Applied Biosystems, Waltham, Massachusetts, US) was used to carry out the DNA amplifications. The PCR conditions for the amplification of the ITS region were set according to Bien et al. (2020). The amplification conditions for the primer pair *Tact1* + *Tact2* were: initial denaturation at 94°C for 10 min; followed by 30 cycles of denaturation at 94°C for 35 s, annealing at 48°C for 30 s and extension at 72°C for 80 s; and a final extension step of 10 min at 72°C. The amplification conditions for the primer pair T1 + Bt-2b were set according to Cabral et al. (2012b) with the exception of a 60°C annealing temperature. A 1% agarose gel was used to visualise the PCR products. The products were sent to Eurofins Scientific Laboratory (Ebersberg, Germany) for sequencing. By using BioEdit Sequence Alignment Editor (v. 7.2.5; Hall 1999), the sequences were checked. Sequences were submitted to GenBank.

2.5 Identification of fungi

Fungal species were identified based on morphological observation and molecular analysis. The BLAST algorithm (<http://www.ncbi.nlm.nih.gov/genbank>, Altschul et al. 1997) was used for fungal taxon determination. The results were re-checked against literature and known cultures for confirmation. BLAST results below a threshold of 98% identity were not trusted to be accurate enough for final determination. Each identification was critically interpreted with emphasis on well curated culture collections such as the Westerdijk Fungal Biodiversity Collection (CBS). In case no definite affiliation was possible to a specific taxonomic level the identification was marked by cf. (confer) to indicate uncertainties.

For strains belonging to *Neonectria*, final determination of the species was based on two separate comprehensive phylogenetic analyses using the ACT and TUB gene regions, respectively, including reference sequences retrieved from GenBank (data not shown). Additionally, for the final determination of strains belonging to the genus of *Eutypa* sp. a phylogenetic analysis based on the ITS results was used.

2.6 Analysis with R

Analysis of the fungal diversity found in this study was conducted using RStudio (V 4.1.2) (R Core Team 2021). The packages used were tidyverse (Wickham et al. 2019), where a distribution chart was created using the function "pie". The packages ggplot2 (Wickham 2016) and ggVennDiagram (Gao 2021) were used to analyse the overlap between the fungi found at each site. Here the function "ggVennDiagram" was used.

3. Results

3.1 Distribution of SBD in German forests

By 31.05.2021, we had received reports of SBD from forests all over Germany with the exception of the federal state of Schleswig-Holstein, where no outbreak has been recorded so far. In total, 403 stands with obvious symptoms of SBD were reported (Fig. 1). Of the 123 evaluated stands in Hesse, 31.71% showed visible symptoms of SBD. At the beginning of 2022, a collection of SBD reports were registered in the WSMP for Hesse.

3.2 Isolated fungi

In total, 4,124 segments of stem tissue increments originating from 50 trees were incubated. From these, 379 mycelial outgrowths were observed and 292 of them were transferred to pure cultures (Online Resource 2). Most fungi grew out between week one and three after incubation of the increment segment. When the original plates were discarded, only a few outgrowths had been observed in the preceding week. Some of the 379 outgrowths were omitted due to obvious repetitions or contaminations. The resulting pure culture isolates were assigned to 91 species and all but 14 could be identified to genus or species level (Table 2). The majority of the wood tissue increments studied (93.8%, $n = 3,163$ studied increment segments) had no visible discoloration or even signs of rotting. From sampling sites in Fulda, Beerfelden, Nidda and Nehnten, 28 of the 80 increments showed visible signs of infection, resulting in 195 increment segments with wood discoloration or rot (6.2%, $n = 3,163$). From those segments yeasts grew out in 78 cases, mycelia in 43 cases (nine species) and no growth was observed in 78 cases. Seven filamentous fungal species isolated from discoloured or decayed wood were identified to genus or species level, namely *Biscogniauxia nummularia* (Bull.) Kuntze, *C. corticale*, *Diaporthe* sp., *Furcaterigmium furcatum* (C. Moreau & Moreau ex W. Gams) Giraldo López & Crous, *Jackrogersella cohaerens* (Pers.) L. Wendt, Kuhnert & M. Stadler, *Leptosillia muelleri* (Duby) Voglmayr & Jaklitsch and *Neonectria* sp. The remaining two species could be assigned to the order of *Agaricales* (NW-FVA 7192) and the family of *Hymenochaetaceae* (cf. *Inonotus* sp. NW-FVA 7019), respectively.

The majority of the isolated filamentous fungi from all samples were *Ascomycota* (79 taxa, 86.81%), nine taxa (9.89%) belonged to the division of *Basidiomycota* and one species was determined to be *Mucoromycota* (1.1%). The remaining two taxa (2.19%) could not be classified due to unsuccessful DNA-extraction, one presumably being a coelomycete fungus. Within the *Ascomycota*, the most frequently observed orders (Fig. 2) were *Pleosporales* (26.58%) followed by *Xylariales* (13.92%) and *Hypocreales* (12.66%). The *Basidiomycota* species were identified as *Agaricales* sp. (NW-FVA 7192), *Coprinellus micaceus* (Bull.) Vilgalys, Hopple & Jacq. Johnson (NW-FVA 6233), *Coprinellus* sp. (NW-FVA 7000), *Hymenochaetaceae* sp. (NW-FVA 7019), *Hypholoma fasciculare* (Huds.) P. Kumm. (NW-FVA 6421), *Porostereum spadiceum* (Pers.) Hjortstam & Ryvarden (NW-FVA 6997), *Serpula himantoides* (Fr.) P. Karst. (NW-FVA 7710), *Stereum* cf. *hirsutum* (NW-FVA 6270), and *Trametes versicolor* (L.) Lloyd (NW-FVA 6271).

Of the 91 species detected, 41 (45%) were isolated more than once and only six species were obtained ten or more times. The remaining 50 species (55%) were only isolated once. Between 0–19 different species were found in the studied woody tissue per tree. On average, 3.3 species were recorded on each tree. From four trees no isolations could be made. None of the identified species were found at all sites and merely 15 out of the 91 species were found at more than one site.

In total, 84.62% of the species were found solely at one site while 63.74% of all isolated species were isolated from one single tree. The top ten species with the highest frequency were *Hymenochaetaceae* sp. (10.6%), *C. corticale* (7.85%), *Cytospora* cf. *populina* (6.48%), *Cytospora* cf. *rodophila* (5.12%), *Thyridium vestitum* (Fr.) Fuckel (4.78%), *Lopadostoma turgidum* (Pers.) Traverso (3.41%), *Xylaria longipes* Nitschke (3.07%), *Penicillium* sp. (3.07%), and *Nectria cinnabarina* (Tode) Fr. (3.07%).

The most abundant species, in regard to both occurrence at the studied sites and continuity, were *C. corticale* (4 sites, 26% continuity, 7.85% frequency), *X. longipes* (4, 14%, 3.07%), *L. turgidum* (3, 10%, 3.41%), *Cadophora prunicola* Damm & S. Bien (3, 6%, 1.02%), *Trichoderma* sp. (3, 6%, 1.02%), *L. muelleri* (2, 12%, 2.05%), *Diaporthe pustulata* Sacc. (2, 6%, 1.02%), *Didymella macrostoma* (Mont.) Qian Chen & L. Cai (2, 6%, 2.05%), and *B. nummularia* (2, 6%, 1.71%).

The number of species isolated at the different sites ranged from 13 to 44. Hence, the fungal communities at the sites studied differed in their species composition and diversity (Fig. 3). In Melsungen, 44 species (48.35% of all isolated taxa) were found and 36 (39.56%) of those occurred exclusively at this site. The most common fungal species in Melsungen, sorted by continuity, were *C. corticale*, *Penicillium* sp., *Cytospora* cf. *rodophila*, *Cytospora* cf. *populina* and *T. vestitum*. *Cryptostroma corticale* was isolated from 60% of the trees studied at this site. Twenty-one species (23.07%) were found in the samples from the stand in Fulda, eleven of which (12.01%) were only found there. The most common species at that site were *X. longipes*, *L. muelleri*, *L. turgidum*, *D. pustulata*, and *Neonectria* sp. *Cryptostroma corticale* was not isolated from any tree in Fulda. At the Beerfelden study site, 19 species (20.9%) were found, 13 of

which (14.29%) were found exclusively at that site. The five most frequently found species were *Arthrinium rasikravindrae* Shiv M. Singh, L.S. Yadav, P.N. Singh, Rah. Sharma & S.K. Singh, *Arthrinium cf. marii*, *L. muelleri*, *C. corticale* and *Lophiostoma carpini* Andreassen, Jaklitsch & Voglmayr. *Cryptostroma corticale* was found in one of the trees studied (10%). 15 species were found in the Nidda samples (16.5%), eight of which (8.8%) were exclusive to that site, namely *C. corticale*, *B. nummularia*, *L. turgidum*, *Hymenochaetaceae sp.*, and an unidentified species (NW-FVA 7196). *Cryptostroma corticale* was isolated from 5 out of 10 trees studied in Nidda (50%). At Nehmten, 13 fungal species (14.29%) were isolated in total, eight of which (8.8%) were found exclusively at that site, including *C. corticale* (10%). At that location only one fungus, *Hypoxylon fragiforme* (Pers.) J. Kickx f., was isolated more than once.

4. Discussion

The observed widespread occurrence of SBD in Germany coincides with information from neighbouring central European countries (Kelnarová et al. 2017; Cech 2019; Queloz et al. 2020). As shown by the SBD reports documented in this study, there are no cases of the disease in the forests of the most northern federal state of Germany, Schleswig-Holstein. This might be due to a more maritime, and thus more humid, climate. When comparing the site conditions of the forest stand where the endophytes were isolated, it can also be observed that the site in Nehmten, Schleswig-Holstein has a different bedrock than the other sites (Table 1). The Forest plot in Nehmten was the only site with a high water storing capacity (WSC). The WSC is highly dependent on the amount of skeleton in the ground since it takes up valuable space that cannot be used for storing water. The sandy stand in Nehmten has a larger soil depth, as well as less skeleton (less than 5%), in the soil compared to the sites in Hesse (Basalt, 10% – 15% skeleton on average, which increases with soil depth). Thus, the site in Nehmten has a higher WSC (Paar et al. 2016). Additionally, the site in Nehmten is influenced by ground water due to being located in a lake region. This indicates that even in drought periods, the plant water availability is more stable in Nehmten, resulting in less plant stress. When comparing climate data for 2018 and 2019 between Hesse and Schleswig-Holstein, the data for 2018 shows that the average summer temperature (AST) in Hesse was 19.6°C, 3.4°C above the average of the reference period (ARP) and the AST for Schleswig-Holstein was at 18.4°C, only 2.6°C above the ARP (DWD 2018b). The difference between the two states was similar for 2019, for Hesse the AST was 19.1°C, 2.9°C above the ARP and for Schleswig-Holstein 17.9°C, which was 2.1°C above the ARP (DWD 2019b). For 2018 and 2019 the precipitation deficit both for the summer period and the entire year was lower for Schleswig-Holstein than for Hesse (DWD 2018b, 2019b). This shows that temperatures in Hesse were in general higher, as well as in relation to the ARP, with less precipitation in comparison to Schleswig-Holstein, indicating potential higher drought stress and thus more favourable circumstances for the outbreak of SBD in Hesse.

The result of Kelnarová et al. (2017) that in a quarter of all studied urban sycamore trees in Prague *C. corticale* could be detected as endophyte was confirmed by our observations of the latent non-symptomatic stage of SBD in forest trees. Our result that 26% of all studied, apparently healthy sycamores, harboured *C. corticale* non-symptomatically, regardless of the occurrence of external symptoms in the studied forest stands or area, lead to the assumption that latent infection with *C. corticale* is widespread in Germany. Therefore the risk of mortality due to *C. corticale* for apparently healthy sycamore trees rises with the increase of years with drought and extraordinary high temperatures, since SBD is triggered by drought and hot temperatures (Dickenson 1980; Ogris et al. 2021). Even though only vital trees with no signs of distress or injury were chosen for our sampling, 35% of the increments exhibited wood discoloration and/or wood rot. In Kelnarová et al. (2017), discoloration was observed in 59% of all sampled trees and was correlated strongly with the presence of *C. corticale*. In this study *C. corticale* was only isolated once from discoloured areas. This might have been the result of analysing vital trees, not focusing on discoloured tissue. The amount of discoloured tissue observed in this study was also rather low and thus not a basis for adequate characterization. Furthermore, direct correlation of discolouration with *C. corticale* is speculative, since the discolouration could have as well been caused by other agents.

The total amount of endophytic species isolated from woody sycamore tissue in this study (91) is significantly higher than in previous investigations were 10–52 different species were detected (Butin and Kowalski 1986; Kowalski and Kehr 1992; Unterseher et al. 2005; Brglez et al. 2020a). This difference in diversity can be explained by the larger sampling size and the greater number of sites studied here in contrast to the other studies.

Many of the fungal species detected in our study were isolated just once, which may indicate a sporadic occurrence or sampling bias since only ten trees per site were studied at one specific height. However, it cannot be ruled out that these fungi occur in other stands as well as in higher abundance and simply were not isolated from the sampled material. Due to the rather small sample size of two or

three increments compared to the entire wood body of the tree, the listed fungi are likely just a small fraction of the fungal community. Additionally, it is to be expected that the composition of fungi might differ within the tree, depending on the host tissue type (Gennaro et al. 2003) and tree age (Halley et al. 1994; Maherali and Klironomos 2007). Furthermore, a possible underestimation of fungal diversity in the studied trees may occur since not all fungi are detectable through standardised culture based methods or in general (Guo et al. 2001; Allen et al. 2003; Unterseher 2007; Muggia et al. 2017). The composition of the forest stands combined with the nutrient and water availability could also be a factor in assessing the differences in fungal diversity per stand. The stand in Nehnten, stocked only with sycamore, had the lowest fungal diversity of all studied sites and at the same time the lowest nutrient availability.

Similar to other studies on endophytes of tree woody tissues (Langer et al. 2021; Singh et al. 2017; Bußkamp 2018; Ghobad-Nejhad et al. 2018), in this study mainly *Ascomycota* (85.17%) and significantly less *Basidiomycota* (9.89%) were isolated. This also corresponds to studies focusing on endophytes colonising tree tissues (Petrini and Fisher 1988; Kowalski and Kehr 1992; Peršoh et al. 2010; Martínez-Álvarez et al. 2012; Sanz-Ros et al. 2015). A reason for the lower frequencies of *Basidiomycota* compared to *Ascomycota* detected in this study might be that fungi belonging to the former often need longer incubation periods in order to grow out from incubated woody tissues (Oses et al. 2008) but since the incubated increment segments were kept for four weeks on nutrient media, it has to be assumed that enough time was given for fungi to grow out.

Sieber (2007) concluded that the fungal communities in the *Aceraceae* are mainly dominated by species belonging to *Diaporthales*. Furthermore, *Pleosporales* and *Xylariales* can be dominant endophytes in angiosperms. The species identified in this study mainly belong to *Pleosporales*, followed by *Xylariales* and *Hypocreales*. Species belonging to the *Diaporthales* constitute only the fourth most common group. In comparable studies focusing solely on woody sycamore tissue (e.g. Butin and Kowalski 1986, Kowalski and Kehr 1992, Unterseher et al. 2005 and Brglez et al. 2020) the most commonly isolated orders include *Diaporthales*, *Helotiales*, *Hypocreales*, and *Pleosporales*. *Diaporthales* did not unequivocally dominate in any of these studies. This discrepancy in the diversity could be explained by the fact that Sieber (2007) focused on different forest trees and endophytes isolated from leaves and woody tissue except roots. The composition of fungal orders inhabiting leaves and woody tissue, as subsumed by Sieber (2007), might not represent the composition solely in woody tissue of *Acer* trees in specific. The fungal community of this and the aforementioned studies were not dominated by a few host-specific species as stated by Sieber (2007). In our study only five species (*C. corticale*, *L. muelleri*, *L. turgidum*, *Penicillium* sp., and *X. longipes*) were found in 10% or more of the examined trees. Only one of these is genus-specific, namely the most abundant, invasive species *C. corticale*. This might be due to the small geographical region that the analysed sites were located in, in comparison to the geographical area covered in the study of Sieber (2007), as well as the diversity of the studied plants or even the differing sampling and isolation methods used.

The ten most abundant fungi were all isolated from healthy wood tissue. Therefore, it must be assumed that all of them exhibit an endophytic life stage, even though three of them were isolated from discoloured tissue once as well (*B. nummularia*, *C. corticale* and *L. muelleri*). *Cryptostroma corticale* appears to exhibit an endophytic life stage, as it was isolated from healthy, as well as from discoloured, wood. This observation is supported by its affiliation to the *Xylariales*, which is an order hosting many fungi with endophytic life stages (Hendry et al. 2002; Bußkamp et al. 2020). *Xylaria longipes* was the second most abundant species in the study and is a known endophyte in a number of different tree species. *Xylaria* species often grow and sporulate on lying deadwood and stumps (Scholtysik et al. 2013). The third most abundant species, *L. turgidum*, belongs to the *Xylariales* as well. *Leptosillia muelleri*, being the fourth most abundant species, belongs to *Xylariales* like *C. corticale* and *Xylaria* sp. and thus can be assumed to have at least an endophytic life stage. *Cadophora prunicola* was only described in 2020 by Bien and Damm (2020) and to the authors' knowledge has not been reported elsewhere since. This is the first study to report *C. prunicola* from *A. pseudoplatanus*, and furthermore it was isolated from healthy tissue. With *C. prunicola* belonging to the *Helotiales*, an endophytic life stage is probable, since several other fungi belonging to the *Helotiales* are known endophytes (Petrini and Fisher 1988; Langer et al. 2021). *Trichoderma* species can occur as endophytes as described by Evans et al. (2003) and belong to the *Hypocreales*, which is a group hosting several other endophytic species as well. *Diaporthe pustulata* was originally isolated from *A. pseudoplatanus* (Gomes et al. 2013). As described in Suryanarayanan (2011), Bußkamp et al. (2020) and Langer et al. (2021), species belonging to the *Diaporthales* can occur as endophytes, making it likely for *D. pustulata* to also have an endophytic stage in its life cycle. The pleosporalean fungus *D. macrostroma* is presumed to have an endophytic life stage as well. Many other fungi in the *Pleosporales* also have endophytic life stages (Langer et al. 2021). *Biscogniauxia nummularia* is a known and widespread multi-host endophyte isolated from several different tree species (Petrini-Klieber 1985; Nugent et al. 2005, (Petrini-Klieber 1985, p.; Chapela and Boddy 1988; Chapela 1989; Bußkamp et al. 20) belonging to the *Xylariales* as well, and very common on beech trees (Chapela and Boddy 1988; Chapela 1989).

The fungus isolated with the highest frequency, but very low abundance, was one belonging to the family of *Hymenochaetaceae*. Even though this species has the highest frequency, it is not the most important or common species since all but one isolate originate from one tree. The respective cores showed signs of white rot and the species was isolated from many pieces of one of the cores.

Detection of some fungal groups, like *Penicillium* sp. or *Trichoderma* sp. can be explained, since these groups have in general a wide host range as well as a wide geographical distribution and are frequently reported in comparable studies (Cotter and Blanchard 1982; Chapela 1989; Ragazzi et al. 1999; Hendry et al. 2002; Baum et al. 2003). Several wood decay fungi were isolated and three species them were isolated from discoloured woody tissue (*B. nummularia*, *Hymenochaetaceae* sp., and *J. cohaerens*).

The composition of fungi isolated in this study differed significantly between the studied forest sites, with very little overlap between the sites. It can be assumed that adding another differing site would reveal an entirely new set of endophytes not recorded in this study. While some of the isolated fungi (29.1%) were already described as associated with maple (see Ellis and Ellis 1985; Butin and Kowalski 1986; Chlebicki 1988; Kowalski and Kehr 1992; Unterseher et al. 2005; Brglez et al. 2020), most were not recorded in the aforementioned articles (70.9%). Only ten of the isolated species (*C. corticale*, *D. pustulata*, *D. rudis*, *D. macrostroma*, *Eutypa maura* (Fr.) Fuckel, *N. cinnabarina*, *N. acerina*, *Petrakia irregularis* Aa, *T. vestitum* and *X. longipes*) were listed for *A. pseudoplatanus* in the USDA fungal database (Farr and Rossman 2022). Three of them were also reported from sycamore in Germany. This further illustrates the current lack of knowledge about endophytes, specifically in woody tissue of *A. pseudoplatanus* in Germany.

Cryptostroma corticale was isolated from four out of five sampled stands. It was found in Melsungen, a site with trees without obvious symptoms of SBD and located more than 30 km away from the next known area of infection. Furthermore, it was detected in Nehnten, however, symptoms of SBD have not yet been reported for the entire federal state of Schleswig-Holstein. Isolation of *C. corticale* in Beerfelden was unexpected, due to the high wood quality and tree vitality of the sampled stand. The detection of *C. corticale* in Nidda was expected, due to obvious symptoms of SBD at the site. In contrast, the fungus was not detected at the stand of Fulda despite of obvious symptoms recognised. Similar to the results of Kelnarová et al. (2017), who also used both a culture and a non-culture based isolation method sampling 112 trees on seven different sites, the number of trees from which *C. corticale* was isolated was lower than expected. The continuity of *C. corticale* in the presented study is 26%, similar to the results of Kelnarová et al. (2017) where the continuity, based on both isolation methods combined was 25%.

The spread of *C. corticale* in its endophytic stage should be further investigated in Germany as well as in Europe in order to estimate the potential risk for *Acer* cultures in this region. The presented data functions as a basis in this endeavour. An increase of negative effects due to climate change, such as rising temperature and insufficient precipitation, need to be expected for the future, and constitute major risks for the forest sector. In the specific case of SBD, the location of *Acer* plantations should be carefully considered in order to increase the potential resilience of the tree. Additionally, the discovery of potential biological control agents against *C. corticale* among the determined endophytes could help improve resilience of sycamore trees against SBD.

Declarations

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Statement of contributions

The study including sampling, lab work and analysis was primarily conducted by R. Schlößer with support from G.J. Langer and S. Bien and supervision of E.J. Langer and G.J. Langer. The first draft of the manuscript was written by R. Schlößer and revised by S. Bien, G.J. Langer and E.J. Langer.

Compliance with ethical standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics approval

Not applicable

Consent to participate

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Consent for publication

Not applicable

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Tables

Table 2 is available in the Supplementary Files section.

Figures

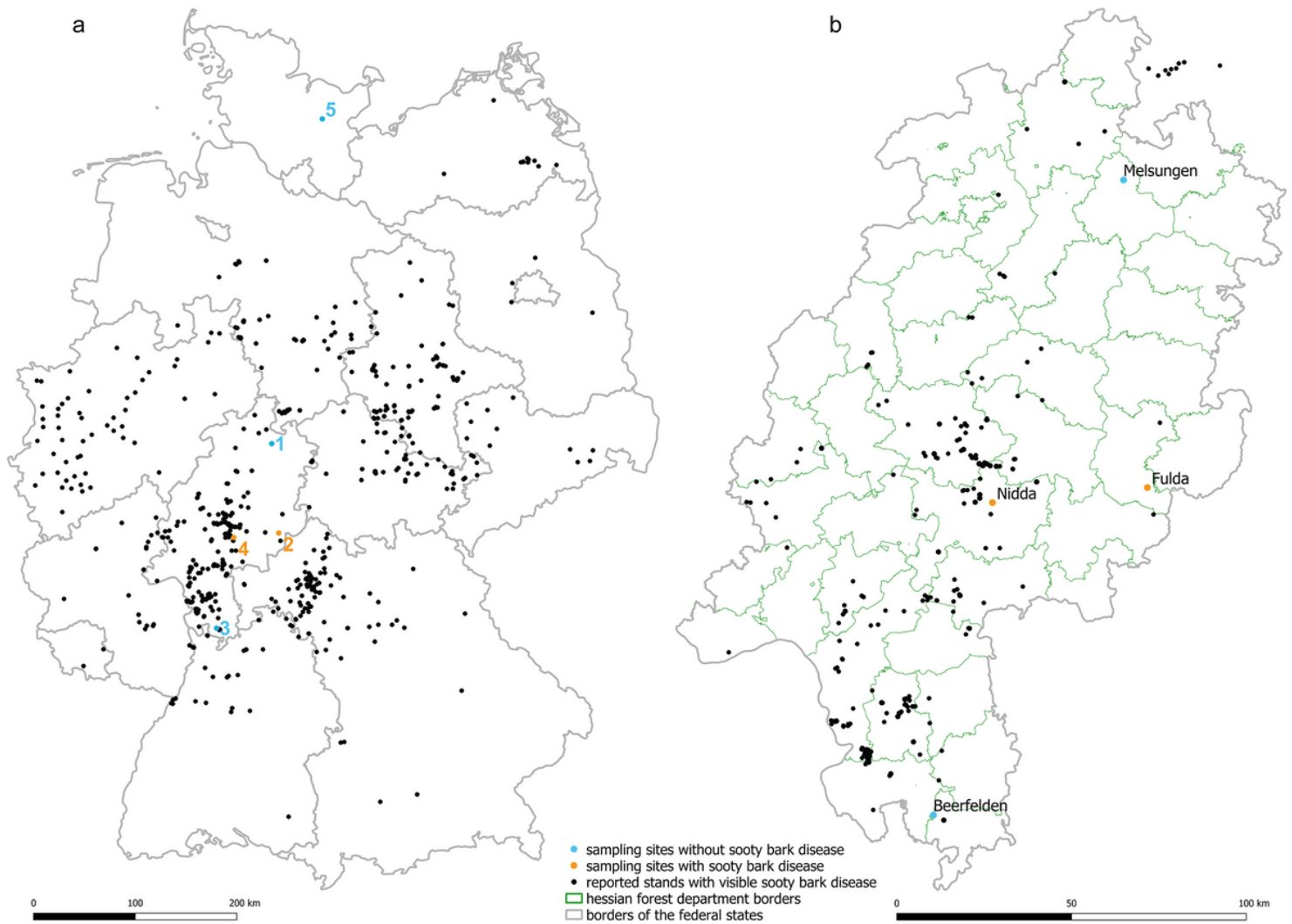


Figure 1

Distribution of SBD **a**: cases of disease in Germany, reported by 31.05.2021, study sites: 1 = Melsungen, 2 = Fulda, 3 = Beerfelden, 4 = Nidda, 5 = Nehmten; **b**: detailed view of the cases in the federal state of Hesse; © GeoBasis-DE / BKG (2021), © HessenForst 2020, last updated: 31.03.2022 (QGIS Desktop 3.22.3).

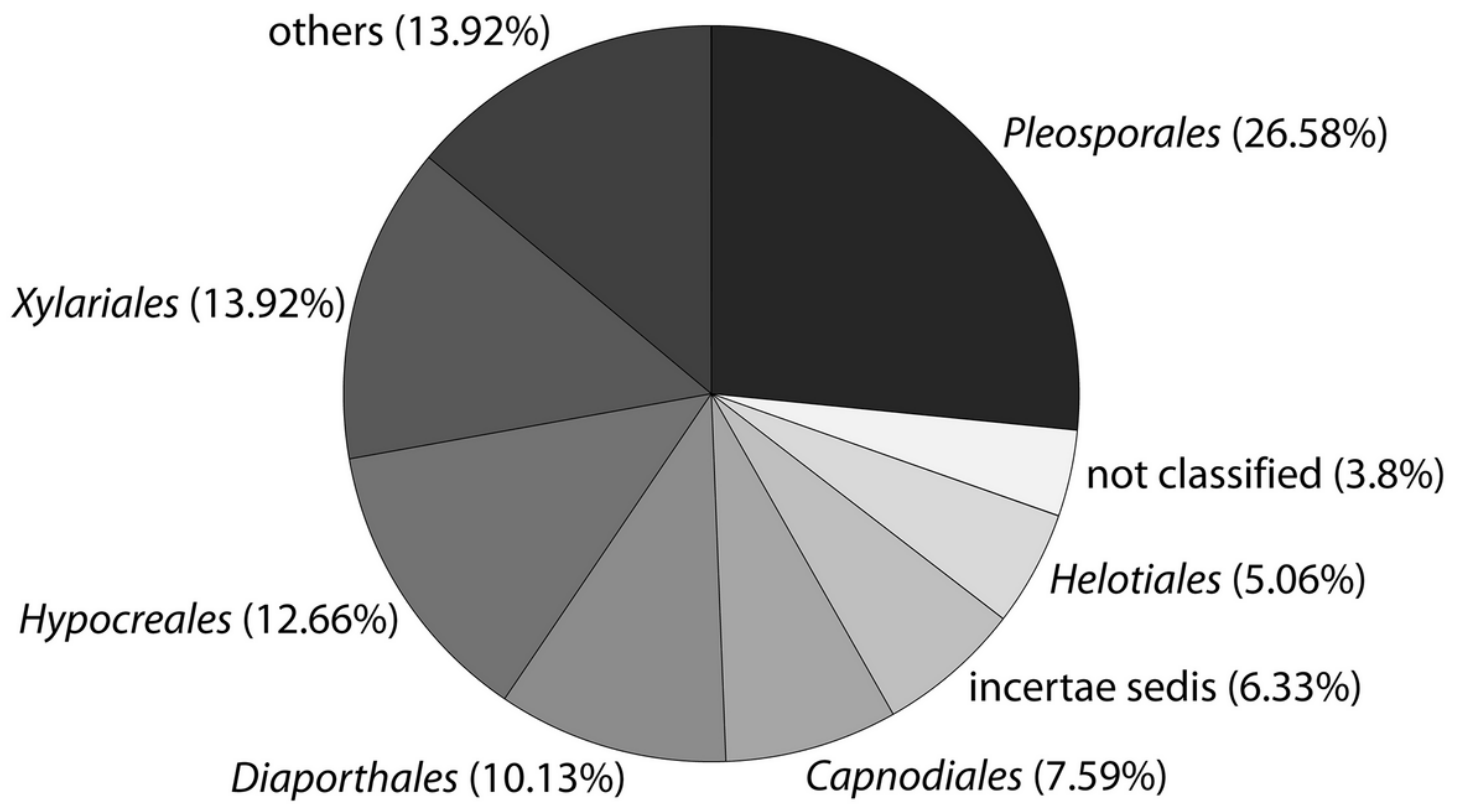


Figure 2

Percentage of isolated endophytic *Ascomycota* orders, n = 79 of the isolated species belonging to the *Ascomycota* (RStudio 4.1.2).

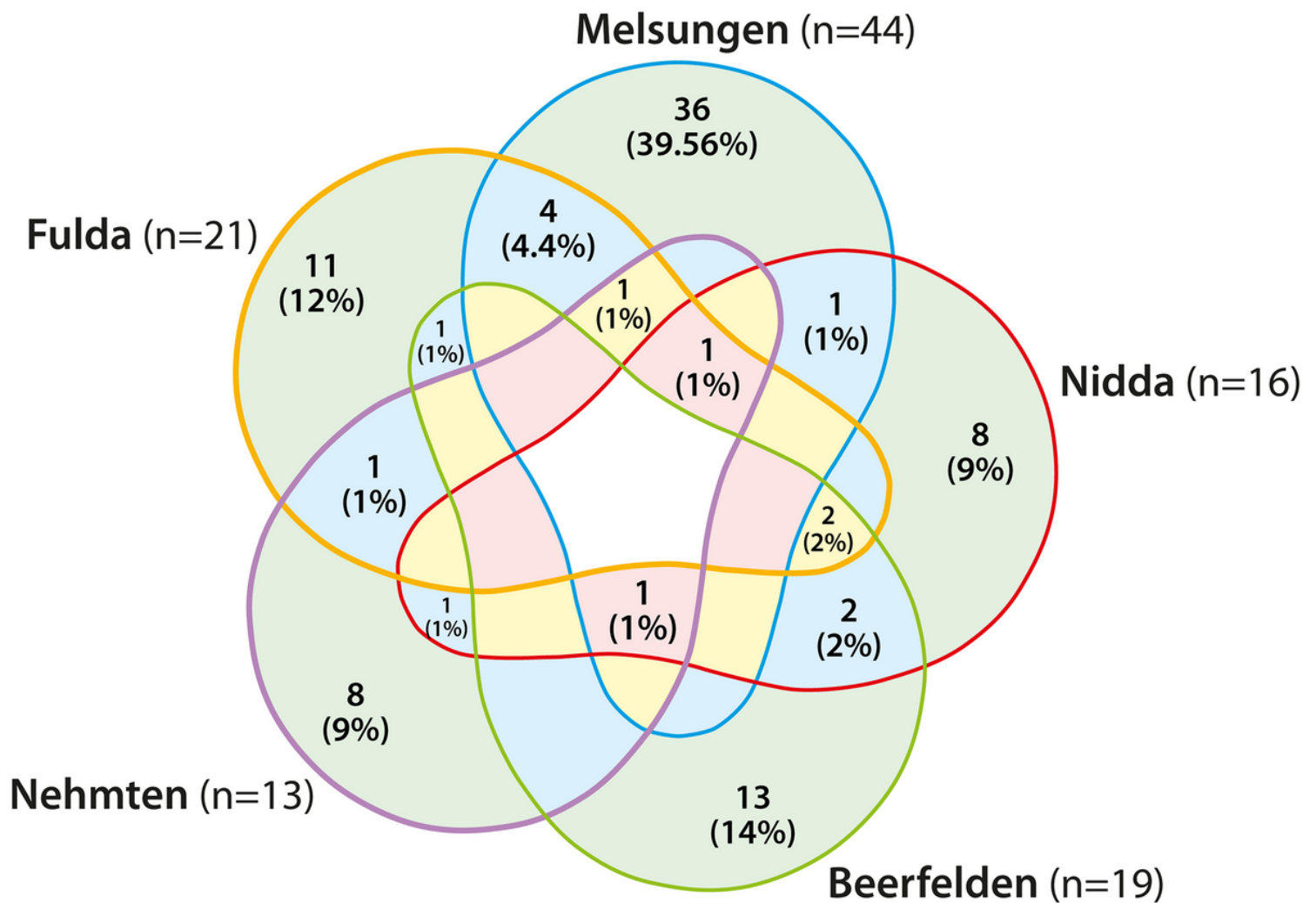


Figure 3

Comparison of the endophytic fungal communities of the different study sites; Overlap between the fungi (absolute number as well as percentage); green filling = fungi isolated just at the respective site, blue = fungi isolated from two sites, yellow = fungi isolated at three sites, red = fungi isolated from four sites (RStudio 4.1.2).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table2.docx](#)
- [OnlineResource1Assessmentofincrementtrees.xlsx](#)
- [OnlineResource2Overviewofisolatedfungi.xlsx](#)