Research Square

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The Opportunistic Pathogen Sphaeropsis Sapinea is Found to be one of the Most Abundant Fungi in Symptomless and Diseased Scots Pine in Central-Europe

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Abstract

Background: The opportunistic and latent pathogen *Sphaeropsis sapinea* is one of the most important forest pathogens on pine. The fungus may cause Diplodia tip blight on several coniferous trees and disease symptoms come visible when trees are weakened by stress, usually related to injuries and drought. This project compares the mycobiome of healthy and diseased Scots pines. Twigs were sampled in June and September 2018 in a German forest stand with varying health status of the sampled Scots pines. Growth of 2017 and 2018 were sampled and cultivable, filamentous fungi isolated and the mycobiome was analysed by high-throughput sequencing (HTS) of the internal transcribed spacer 2 (ITS2) region.

Results: A PERMANOVA analysis confirmed that the mycobiome community composition significantly differed between growth years ($p < 0.001$) and sampling time ($p < 0.001$) but not between healthy and diseased trees. Higher amount of *S. sapinea* was observed in June and the growth year 2017. Besides yeasts which were neglected, 23 ascomyceteous fungal endophytes were isolated. *S. sapinea* was the most common endophyte isolated and the second common in HTS data. It was highly abundant in symptomless (healthy) trees.

Conclusion: Results highlights the ability of *S. sapinea* to accumulate unnoticed before disease outbreaks, implementing the sudden threat for Scots pines in the future.

Background

The distribution of forest pathogens and pests is drastically increasing world-wide. Changes in climatic conditions provide the establishment of new fungal pathogens in previously non-optimal environments. This leads to severe declines of native tree species, which are affected with new threats to trees defence system. Outcomes are losses to valuable forest biodiversity and forest industry. Fungal species are among the most damage causing agents due to rotting timber or leading to deaths of mature trees [1]. However, fungal pathogens have niche competitors in the new environments, which in theory could be used as antagonists against them [2]. Trees do not grow alone – they are accompanied with a huge and yet undiscovered mycobiome. Newest molecular technologies such as high-throughput sequencing (HTS) open up new opportunities to understand the nature behind host-pathogen-mycobiome interactions that are competing inside the plants tissue. In that sense, the aim of this study was to monitor Diplodia tip blight, a severe tree disease of Scots pine caused by *Sphaeropsis sapinea,* and its host's mycobiota between symptomless and diseased trees.

The pathogen: *Sphaeropsis sapinea*

Sphaeropsis sapinea (Fr.) Dyko & Sutton Botryosphaeriaceae, Botryosphaeriales Theiss. & Syd. (most common synonym: *Diplodia sapinea* (Fr.) Fuckel) is the causal agent of Diplodia tip blight (Sphaeropsi*s* tip blight) of conifers. The anamorphic fungus has been first described as *Sphaeria sapinea* Fr., collected in Sweden from *Abies* sp. and *Pinus* sp. by Fries [3]. Within its life cycle, *S. sapinea* has different trophic stages [4]. It can live asymptomatically as an endophyte in its host tree [5, 6], while being a latent and opportunistic pathogen [7, 8] or/and saprophyte [9]. In combination with stress-inducing factors, such as drought, hail, extreme temperatures or mechanical wounding [10, 11] *S. sapinea* may become pathogenic, leading to a disease outbreak [12, 13]. Conidia can enter the host through lenticels and stomata [14, 15] or wounds caused by insects or hail [8]. Further, it is discussed that spores might enter through the needle base or non-lignified shoots [16–20]. Infections are mainly airborne and conidia transported by wind and water droplets or carried by vectors like bark beetles (Coleoptera: Scolytinae; [21]) or *Hylobius abietis* [22].

Diplodia tip blight (disease development)

Although the origin of *S. sapinea* is still unknown, it was most likely introduced to new regions with the movement of seeds or symptomless host material [12, 23, 24]. Ghelardini et al. [25] stated that cryptic and latent pathogens, like *S. sapinea*, are one of the most important drivers of emerging fungal diseases in forests. Already decades ago in the southern hemisphere, *S. sapinea* caused devastating diebacks in the genus *Pinus* [8, 26, 27]. *S. sapinea* is also a threat to the Northern Hemisphere, as indicated by several reports of new outbreaks in North America [e.g. 12, 23, 28], Central-Europe [13, 29, 30] and Southern Europe [e.g. 31, 32]. A sudden disease outbreak and invasion to Northern Europe was observed in Estonia by Hanso and Drekhan [33] and in Sweden by Oliva et al. [34] and Brodde et al. [35]. In Finland, *S. sapinea* was found on cones in 2015 and 2016 [36]. Recently it was also found as latent endophyte in shoots of Scots pine in Finland [6]. Due to the changes in climatic conditions with increasing temperatures, the growth of *S. sapinea* is favoured by a warmer climate [7]. Because of the endophytic stage of *S. sapinea* the accumulation of this pathogen before disease outbreaks can happen unnoticed [35]. However, it is not confirmed whether the species is native to Europe [37].

The host: conifers and their health status

Several conifer species, especially the genus *Pinus*, are the main hosts [38] for *S. sapinea*. Over 33 *Pinus* spp. are known to be susceptible [9]. In Europe, native pine species as Austrian pine (*Pinus nigra* J.F. Arnold), Mountain pine (*Pinus mugo* Turra) and Scots pine (*Pinus sylvestris* L.) are most susceptible [29, 39, 40]. Recently, *S. sapinea* has also been found as endophyte in broadleaved trees such as *Fagus sylvatica* [41, 42]. As the local environments are changing, Scots pine trees in Germany have to face the loss of vitality due to expected frequency of droughts, exacerbated by long lasting periods of high temperature and high solar radiation [43]. Scots pines stressed by drought are observed to be especially sensitive to disease outbreaks when additionally damaged by hail [8, 44]. Typical disease symptoms of Diplodia tip blight are brown and short-needled, dead current-year shoots (Fig. 1a) [45], resinous cankers on main stems and branches (branch and bole canker), dieback and misshapen tops. *S. sapinea* (Fig. 1b) can also cause death of cones, seedling blight and sapwood staining [46], damping off and collar rot of seedlings, and root diseases [9], all of which may lead to the death of the entire tree (Fig. 1c).

Characteristics and functions of a tree's mycobiome

Forest trees host divergent varieties of microbes and maintain dynamic balanced relationships with them. The hidden diversity of the mycobiome, defined as all fungal organisms that live inside (endophytes) and across (epiphytes) the host trees tissue, can have an extremely important function as the diverse composition of the undiscovered fungi might enhance the fitness of not only individual trees, but also of the whole forest ecosystem [47, 48]. In that sense, it is possible that differences in the mycobiota of healthy and sick trees might influence the processes leading to disease outbreak and different varieties of disease symptoms. It is therefore important to highlight the ecological and evolutional non-static nature of the host tree's-fungal interactions and the influence of the environment on the outcome of these interactions [49]. The relationships of the tree's mycobiome to its host tree could vary from latent commensalism to mutualism or pathogenic infections [50–53]. Endophytic fungi may interact with their host in a mutualistic symbiosis, which can play important roles in plant nutrition, nutrient cycling, growth, and health [51–54]. With the ability to maintain multiple balanced antagonisms with the host and with the other microbial competitors, endophytes grow asymptomatically in their hosts [57]. This ability could be due to the modulation of host phytohormones, e.g. intrinsic concentrations of jasmonic and salicylic acid that has followed by endophytic colonization [58]. Many studies support the hypothesis that fungal endophytes may enhance the tolerance of the host tree to fungal pathogens [e.g. 2, 59–68]. Those findings are extremely important, as in future, there might be a possibility to favor the use of beneficial endophytes that can act as biocontrol agents against pathogens and in this case against S*. sapinea*. Indeed, endophytes are increasingly being considered or exploited for integrated pest management (IPM).

Yeasts might play a significant part of the mycobiome of pine twigs as compared to endophytes, epiphytes (phylloplane and bark community) or saprophytes in temperate regions [69]. Beside endophytic bacteria and filamentous fungi, yeasts are a unique subset of the symbiotic microflora within plants [70]. Endophytic yeast may contribute to the increased growth and health of the host tree by producing plant hormones and other factors [70]. Since yet, there is only a limited knowledge but yeasts might have different functions in the decomposition of plant materials. They may actively produce hydrolytic enzymes or work as transient fungi that use products resulting of the decay by other organisms like filamentous fungi and bacteria [71].

High-throughput sequencing analysis for studying mycobiomes

Exploring the mycobiome of a plant unravels the large diversity of fungi inhabiting their host. This includes the indigenous and exogenous fungal organisms, cultivable and uncultivable fungi, symbionts and pathogens - the entire range of fungal classes. Still, a limited number of fungal organisms is identified. High-throughput sequencing (HTS) analyses provide the opportunity to explore a host's mycobiome in its most efficient way.

According to Hawksworth and Lücking [72], the fungal kingdom contains up to 3.8 million species. Effective methods, such as HTS, may provide closer discovery about the quantity and species identification of a plant's mycobiome. HTS methods can be divided into second and third generation sequencing [see 73]. The newest technologies of third-generation sequencing platforms function at the level of single molecules and offer much higher read lengths than the earlier generations. This aspect makes them useful in metabarcoding and community analyses [73]. At present, MiSeq Illumina sequencing is commonly used for metabarcoding studies of fungi, and the paired-end approach covers amplicons of up to ~550 bases in length. It is also the principal platform for conducting metagenomic and metatranscriptomic analyses on the basis of fragmented DNA or complementary DNA [73].

The Aim Of This Study

We compared the mycobiome of pine twigs with varying health status degrees in a German Scots pine stand to see potential differences in the compositions of the fungal communities of symptomless (healthy) and diseased trees (increasing amount of symptoms). Bußkamp et al. [30] performed the isolation of 103 outgrowing endophytic fungal species of Scots pine twigs. This approach will leave out non-cultivable fungi and yeasts. HTS methods are used in many other studies to determine the fungal assemblages in their hosts [73–76]. Therefore, we determined the mycobiome by two methods: HTS of the internal transcribed spacer 2 (ITS2) region by MiSeq Illumina sequencing and the culture-based isolation method from the same trees. We compared the mycobiome community structure between different growth years (2017 and 2018), disease classes (0–5) and sampling time (June and September). The plant material chosen (to compare the mycobiome) were asymptomatic pine twigs. As *S. sapinea* enters the host through stomata or wounds and grows into the shoots, it can be assumed that the fungus accumulates mainly in the twigs.

Results

Fungal isolates retrieved by culture-based method versus HTS method

From 228 twigs (including shoots of the years 2017 and 2018, collected from 35 trees) chosen for culturing study 1358 segments (June: 740, September: 618) were plated, resulting with 1425 outgrowing fungi. Due to the difference in length of between the shoots 2017 and 2018, various numbers of segments (3–9) per shoot were studied. Besides yeasts, which were neglected (1.61% of outgrowing fungi), unidentified ascomycetes (1.4% of outgrowing fungi) and *Penicillum* spp. (0.14%), the outgrowing mycelia were assigned to 23 morphologically different species (Table 2, Fig. 2).

Table 2
Taxa isolated with the culture-based method.

Taxon	Author	Frequency (No. of isolates / total No. of isolations (%)	GenBank accession number (this study)
yeasts		1.61	not cultivated
Fungus spp. ascomycetous		1.40	not cultivated
Penicillium spp.		0.14	not cultivated
Alternaria alternata	(Fr.) Keissl.	0.63	MT790311
Biscogniauxia mediterranea	(De Not.) Kuntze	0.14	MT790312
Biscogniauxia nummularia	(Bull.) Kuntze	0.35	MT790313
Botrytis cinerea	Pers.	0.14	MT790314
Desmazierella acicola	Lib.	1.54	MT790315
Diaporthe sp.		0.91	MT790316
Epicoccum nigrum	Link	0.49	MT790317
Hypoxylon fragiforme	(Pers.) J. Kickx f.	0.14	MT790318
Jugulospora rotula	(Cooke) N. Lundq.	0.07	MT790319
Microsphaeropsis olivacea	(Bonord.) Höhn.	6.95	MT790320
Nemania serpens	(Pers.) Gray	0.14	MT790321
Pezicula eucrita	(P. Karst.) P. Karst.	0.21	No PCR product
Pezizomycetes sp.		0.14	Strain died
Phacidium lacerum	Fr.	0.07	not cultivated
Preussia funiculata	(Preuss) Fuckel	0.07	MT790322
Pseudocamarosporium brabeji	(Marinc., M.J. Wingf. & Crous) Crous	0.07	MT790323
Pyronema domesticum	(Sowerby) Sacc.	0.21	MT790324
Rosellinia sp.		0.21	MT790325
Sordaria fimicola	(Roberge ex Desm.) Ces. & De Not.	0.35	not cultivated
Sphaeropsis sapinea	(Fr.) Dyko & B. Sutton	59.44	MT790326, MT790327
Sydowia polyspora	(Bref. & Tavel) E. Müll.	18.53	MT790328
Truncatella conorum-piceae	(Tubeuf) Steyaert	7.86	MT790329
Therrya fuckelii	(Rehm) Kujala	0.07	MT790330

The mean number if isolated strains of a single shoot varied between 4.95–8.3 strains over all disease classes and in both sampling times (Table 1). The mean number of isolated taxa from varied between 3.4–5.7 species per tree (Table 1). All filamentous species observed were assigned to Ascomycetes. Isolates presented classes Sordariomycetes (9 species, 39.1% of the 23 identified species, 10.2% of all outgrowing fungi), Dothideomycetes (7, 30.4%, 77.6%), Leotiomycetes (4, 17.4%, 0.5%), Pezizomycetes (3, 13.0%, 1.9%). Most abundant species observed as isolate, was *Sphaeropsis sapinea* (847 isolates, 59.4% of total of outgrowing fungi) followed by *Sydowia polyspora* (264, 18.5%), *Truncatella conorum-piceae* (112, 7.9%), *Microphaeropsis olivacea* (99, 7%), and *Desmazierella acicola* (99, 1.5%). All other species were isolated with frequency less than 1%: *Alternaria alternata, Biscogniauxia mediterranea, Biscogniauxia nummularia, Botrytis cinerea, Diaporthe* sp., *Epicoccum nigrum, Hypoxylon fragiforme, Jugulospora rotula, Microsphaeropsis olivacea, Nemania serpens, Pezicula eucrita, Pezizomycetes* sp., *Phacidium lacerum, Preussia funiculate, Pseudocamarosporium brabeji, Pyronema domesticum, Rosellinia* sp., *Sordaria fimicola*, and *Therrya fuckelii.*

Ph. lacerum was only isolated in June whereas *E. nigrum, B. mediterranea, Bo. cinerea, H. fragiforme, N. serpens, Py. domesticum*, and *Ps. brabeji* were only isolated in September. Half of the 23 identified species (65%, Fig. 3) were detected also from HTS data. These included *A. alternata, Bo. cinerea, E. nigrum, Th. fuckelii, M. olivacea, N. serpens*, *P. eucrita, Ph. lacerum, Ps. brabeji, S. sapinea*, *Sy. polyspora*, and *T. conorum-piceae* (Supplementary table 1).

The manually categorization into trophic levels based on authors expertise and literature assigned the isolated endophytes as follows: 26% pathogenic on conifers (*T. conorum-piceae, Bo. cinerea, Diaporthe* sp., *Rosellinia* sp., *S. sapinea, and Sy. polyspora*); 26% typical saprophytes *(D. acicola, Pe. eucrita, Ph. lacerum, Ps. brabeji, Py. domesticum*, and *Th. fuckelii*, except from *Ps. brabeji* this species are occurring usually on needles or branches of pine); 17% typical

hard wood colonizer with lifestyles from endophytic, parasitic to saprophytic (*B. mediterranea, B. nummularia, H. fragiforme*, and *N. serpens*); 17%, typical generalist with various lifestyles but often saprobic (*A. alternata, E. nigrum, M. olivacea*, and *S. fimicola*); 9% coprophilous species, usually living saprobic on soil, dung or plant debris (*J. rotula and Preussia funiculata*). The following wood-decay fungi were identified: *B. mediterranea, B. nummularia, and H. fragiforme*. Except from *Diaporthe sp., P. funiculata*, and *Th. fuckelii* all other isolates filamentous fungi were identified as typical endophytes of Scots pine twigs in the sense of Bußkamp et al. [30].

With HTS, altogether 11684725 reads were received from 95 samples after data cleaning. Average number per sample was 122997 reads (min 42537 reads, max 864376 reads). The reads were assigned to 1233 OTUs (Supplementary table 1, Fig. 5). Most abundant OTU in HTS was *S. polyspora* (2537542 reads, 22%), followed by *S. sapinea* (1958770 reads, 17%) and *T. conorum-piceae* (508355 reads, 4%). *M. olivacea* abundance was found to be high (197702 reads, 2%) as well. The variation of reads was high for *S. sapinea* (average 20619 reads with STDV 111375), followed by *S. polyspora* (average 26710, STDV 56506) and *T. conorum-piceaea* (5351 and STDV 5715) highlighting the abnormal distribution of the data.

The observed reads (Fig. 4) represented Ascomycota (541 OTUs, 44%), Basidiomycota (311 OTUs, 25%), Chytridiomycota (13 OTUs, 1%), Glomeromycota (3 OTUs, < 1%). Additionally, two OTUs of Olpidiomycota (3 OTUs, < 1%), Zygomycota (1 OTU, < 1%), and 367 OTUs (30%) remained unassigned (Supplementary table 1). OTUs in Ascomycota could be assigned to Dothideomycetes (195 OTUs, 16%), Eurotiomycetes (69 OTUs, 6%), Leotiomycetes (59 OTUs, 5%), Sordariomycetes (39 OTUs, 3%), Lecanoromycetes (39 OTUs, 3%), Orbiliomycetes (10 OTUs, almost 1%), Incertae sedis (4 OTUs, < 1%), Taphrinomycetes (4 OTUs, < 1%), Arthoniomycetes (2 OTUs, < 1%), Saccharomycetes (2 OTUs, < 1%), and Pezizomycetes (1 OTU, < 1%).Similarly, in Basidiomycota OTUs presented Tremellomycetes (83 OTUs), Agaricomycetes (49 OTUs, 4%), Cystobasidiomycetes (44 OTUs, almost 4%), Microbotryomycetes (38 OTUs, 3%), Exobasidiomycetes (32 OTUs, almost 3%), Agaricostilbomycetes (23 OTUs, 2%) and Pucciniomycetes (10 OTUs, almost 1%). Also, wood-decaying fungi, such as OTU1203 (Vuilleminia sp.) and OTU1168 (*Stereum* sp.) were observed. In Chytridiomycota all OTUs observed were assigned to Chytridiomycetes (10 OTUs), in Glomeromycota to Glomeromycetes (3 OTUs), and in Zygomycota in Mucoromycetes (1 OTU).

With FunGuild script we could assign HTS data to 440 OTUs to trophic mode. After manual curation trophic modes were assigned to endophytes, epiphytes, plant pathogens and wood-decay fungi (Supplementary table 1). Possible true endophytes were detected as 28 OTUs and epiphytes 20 OTUs. Plant pathogens were assigned to 84 OTUs and wood-decay fungi to 12 OTUs.

Most of the OTUs gained by HTS could by manually categorized into trophic levels or lifestyles based on authors expertise and literature (Fig. 4, Supplementary table 1). 59 OTUs could be assigned to the black yeasts including rock inhabiting fungi or black yeast-like (e. g. OTU7, OTU6, OTU11, and OTU23). Therefore it is likely that these OTUs had an epiphytic source. 17 OTUs were ascomycetous non-black yeasts (e.g. OTU124, OTU555, OTU690: *Taphrina* sp., OTU498: *Taphrina* sp., OTU942: *Debaryomyces* sp.). Some of the latter OTUs may have been endophytic like *Debaryomyces* sp. because species of this genus are typical endophytic plant yeasts [77, 78]. Other OTUs like *Taphrina* sp. seem to have an epiphytic source, because they are obligate none-pine host-specific parasites. 5 OTUs were identified as ascomycetous, olive-brownish pigmented hyphomycetes with yeast-like, growth when young and later producing chlamydospore-like structures (e.g. OTU102: *Neophaeococcomyces catenatus* (de Hoog & Herm.-Nijh.) Crous & M.J. Wingf.). 205 OTUs could be assigned to basidiomycetous yeasts, yeast-like and pleomorphic Basidiomycota with yeast stages including smuts. A big part of this OTUs may have an epiphytic source because, these taxa are non-pine host-specific parasites such as OTU405, OTU364, OTU411: *Tremella* spp., OTU1263: *Septobasidium* sp., or smuts like OTU962 and OTU1256. Not a small proportion of the identified basidiomycetous yeast species (e.g. OTU661, OTU1241, OTU802) belong to the group of typical endophytic yeast genera, such as *Cryptococcus* Vuill. (Tremellales, Agaricomycotina), *Rhodotorula* F.C. Harrison, and *Sporobolomyces* Kluyver & C.B. Niel (both Sporidiobolales, Pucciniomycotina) [77, 78]. 26 OTUS were assigned to the Exobasidiaceae, which usually form colonies with single-celled conidia but without hyphae. Members of this basidiomycetous Family are commonly none-pine host-specific plant pathogens [79]. In total 312 (25%) of all detected OTUs with HTS may represent species with yeast or yeast-like stages. 35 OTUs could be classified as filamentous Basidiomycota including three ectomycorrhizal fungi, whereas for 48 basidiomycetous OTUS where no further assignment to trophic stage or lifestyle was possible. The ectomycorrhizal fungi (OTU177, OTU896, and OTU1088: *Laccaria* spp.) can be assumed to have an epiphytic source as symbiotic, root associated species. 274 OTUs were assigned to Ascomycota growing with mycelia, excluding species with probably epiphytic source such as lichens or lichenicolous fungi (38 OTUs), fungicolous or obligate non-pine parasitic fungi (6 OTUs) or ascomycetous sooty molds (2 OTUs). For 125 ascomycetous OTUs where no further assignment to trophic stage was possible, as well as for 372 OTUs which represent Fungi with no significant similarity to sequences in database. Usually species of Chytridiomycetes (10 OTUs) are inhabiting soil, fresh water, and saline estuaries or are parasitic on e.g. amphibians. Therefore it is assumed that chytrid OTUs had an epiphytic source as well as the three Glomeromycota (OTU359, OTU437, and OTU42) which are arbuscular mycorrhizal fungi.

Disease class

Even the composition of disease class 0, in isolate data, is dispersed more alone (Fig. 5A) no statistical difference with PERMANOVA analysis ($p = 0.062$) between disease classes was observed. Based on the “indispecies” analysis *M. olivacea* was statistically different ($p = 0.0001$) in disease class 0 (isolate data). The diversity indexes, Simpson ($p = 0.0625$) and Shannon ($p = 0.135$), were not statistically different in cultivable data. PERMANOVA analysis ($p = 0.141$), permutation test and visualization of HTS data did not show any statistical differences in grouping of OTUs (Fig. 6B). Similarly, the diversity indexes (Shannon $p = 0.871$, Simpson $p = 0.826$) were not statistically different between disease classes. This indicates that species diversity in a disease class is similar (abundance and evenness of the species present) in HTS data. The PERMANOVA comparison was made also for HTS reads observed only in sampling time June ($p = 0.25$) or September ($p = 0.367$) confirming no statistical difference were observed between disease classes. Similarly, the composition of plant pathogens was not statistically different between disease classes ($p = 0.699$). The comparison of these groups confirmed the null hypothesis that the centroids and dispersion of the OTUs and diversity indexes are equivalent for all groups.

In disease class 0 from defined trophic modes, based on FunGuild results, we could assign to pathotrophs 55 OTUs, pathotroph-saprotrophs 98 OTUs, pathotroph-saprotroph-symbiotroph 114 OTUs, pathotroph-symbiotroph 15 OTUs, saprotrophs 65 OTUs, saprotrophs-symbiotroph 3 OTUs and symbiotrophs 24 OTUs. The averages of each group were not statistically different from each other’s (Kruskall-Wallis test, $p = 0.361$).

Sampling time

PERMANOVA analysis showed that the species/OTU composition was different between June and September in both isolate (p = 0.001) and HTS (p = 0.001) data (Figure 6). In isolate data both Shannon (p = 0.00513) and Simpson (p = 0.00826) diversity indices were statistically different indicating higher diversity in September. Diversity indices (Shannon p = 0.557, Simpson p = 0.225) were not statistically different between sampling times in HTS data. *S. sapinea* were statistically higher in June in HTS data set (p = 0.001). *M. olivacea* (p = 0.0001), *T. conorum-piceae* (p = 0.0001) and *E. nigrum* (p = 0.0486) were statistically higher in September based on isolate data. For *S. polyspora* no statistical difference between sampling time was observed.

Year of the growth

The species composition was statistically different in isolate data (p = 0.001) and the composition of OTUs were statistically different (p = 0.001) in HTS data between year of the growth (2017 vs 2018) (Fig. 7). Diversity indices (Shannon, p = 3.76e-05; Simpson p = 5.67e-07) for isolate data were significantly different, indicating higher diversity in 2018. Similarly, diversity indices were significantly different for HTS data (Shannon, p = 2e-16; Simpson's, p = 1.44e-13), indicating higher diversity in 2018.

1. *polyspora* (p= 0.0001) and *S. sapinea* (p= 0.0008) were found to be statistically different between growth years from HTS data. Similarly, in isolate data *S. polyspora* (p = 0.0001) was statistically different between growth years. In HTS data set *S. polyspora* abundance was higher in the growth year 2017 (both sampling times) and in isolate data year 2018.

Detection of *S. sapinea*

Altogether 842 (59.44 % of all outgrowing fungi) *S. sapinea* strains were isolated from 228 twigs. The number of isolates were not statistically different between disease classes (p = 0.0894), time of the year (p = 0.459) or time of the sampling (p= 0.0587) (Fig. 8) in culture-based isolation data.

PERMANOVA analysis with HTS data showed that *S. sapinea* reads were statistically different between sampling times (p = 0.001), growth years (p = 0.001), and disease classes (p = 0.043). The number of *S. sapinea* reads (HTS data) were higher in June and in growth year 2017. Kruskall-Wallis test for HTS data showed that disease class 0 was different from disease classes 2, 4 and 5 (Fig. 9a). Similarly, disease class 3 was different from 2 and 4 (Fig. 9a). Four outliers, all from June 2017 samples, were detected from HTS data (disease class 1 = 132497 reads, disease class 2 = 86340 and 804269 reads; disease class 4 = 730005 reads). After removing these outliers PERMANOVA analysis showed differences between sampling times (p = 0.001), growth years (p=0.012) and disease classes (p = 0.019). The numbers of reads were higher in June and in growth year 2017. Kruskall-Wallis comparison showed that disease classes 2 and 4 (the number of reads) were statistically higher from disease classes 0 and 3 (Fig. 9b). After removing outliers, the average number of reads did not differ statistically between disease classes 0 and 5.

Discussion

Culture-based isolation method and HTS accomplish each other

Selection of methods is crucial for studies aiming at providing the full range of an organism's microbiome or as in this study, the mycobiome of pines' twigs. The large number of detected OTUs with HTS was to be expected, as high fungal community quantities have been described before, outstanding in their diversity of morphologies and trophic strategies [74].

The assemblage of fungi detected with the cultivable method only detects fungi with the ability to metabolize the provided nutrient medium, which are fast enough to grow out of their wood piece in the given time of the experiment and which are not antagonized in their tissue by surrounding filamentous fungi "on their way" growing out to the surface. Experience, thorough design of the method and careful handling not to oversee outgrowing fungi, as it was guaranteed in this study to our best knowledge, certainly increases the likelihood to detect the fungi growing in the observed samples. Except *Diaporthe* sp., *P. funiculata*, and *Th. fuckelii*, all other 20 fungi isolated within this study were also found in a previous study by Bußkamp et al. [30], where 103 fungal species from 25800 Scots pine twigs segments (in comparison to 1358 segments from this study) were identified. Four other *Diaporthe* and two other *Preussia* species were identified by Bußkamp et al. [30]. *Th. fuckelli* is a typical endophyte of Scots pine and occurred in the natural distribution area of its host [80]. Usually it is fruiting on dead branches and it is assumed to be member of the fungal self-pruning community of pine [81].

HTS method on the other hand includes not only endophytes, but also epiphytes and non-cultivable species including yeasts. Nevertheless, the methods proved to accomplish each other: surprisingly, only 65 % of the identified species from the cultivation method were found in the HTS data. Eight species *B. mediterranea, B. nummularia, H. fragiforme, and Rosellinia sp.* (all Xylariales, Sordariomycetes), *J. rotula* (Sordariales, Sordariomycetes), *D. acicola* and *Py. domesticum* (both Pezizales, Pezizomycetes), and Pezizomycetes sp. were not detected by HTS (Fig. 5). Species of Sordariomycetes (39 % of all isolated species in this study by classical method) are common endophytic fungi in plant tissues and comprised 31% of all isolations of Scots pine twigs in the study by Bußkamp et al. [30] or 32% of all isolations from pine branches in studies by Sanz-Ros et al.[82]. *D. acicola* is a typical saprophyte of Scots pine needles and endophyte of Scots pine twigs [30]. *Py. domesticum* is a pyrophilous cup fungus, occurring on burnt or sterilized soils. It typically fruits within a few weeks after a burn [83]. This indicates that both methods are necessary to provide the mycobiome, especially endophytes of an organism. In contrast to our theory that HTS would detect all fungi found in the sequence data from the amplicon method, roughly half of the species were not detected. Indeed, still the choice of primers and databases proves to be the bottleneck for the discovery of all species [73, 84]. The advantage of the culture-based method is the gain of living cultures, which could be tested regard, virulence, antagonism and ecological relevance and function.

Main species of *Pinus* twigs

The main species/OTUs were detected with both methods, but rarer ones only with HTS methods. As confirmed from studies by Bußkamp et al. [30], some of the isolated endophytes of pine twigs may play a role as decomposer or weak pathogens of the host twigs and needles, e.g. *S. sapinea*, *T. conorum-piceae*, *D. acicola, Ph. lacerum,* and *Peniophora pini* (Schleich. ex DC.) Boidin. For the most of the isolated endophytes no significant function is known. Endophytic fungi in twigs of *P. sylvestris* with culture based methods were analyzed by several authors in the past, e.g. [44, 82, 85–88] and the number of detected species varied between 10 and 103. Regularly isolated species are *Sy. polyspora*, *M. olivacea*, *S. sapinea*, *D. acicola* and *Pezicula* spp. Fungi with ubiquitous or generalist lifestyle including *Alternaria, Aspergillus, Cladosporium, Epicoccum, Sordaria, Phoma, Penicillium, Phomopsis, Pestalotiopsis, Xylaria, Nigrospora* also play a role. In contrast to the HTs method, wood decaying fungi were not isolated by the culture-based method in this study. Typical wood decaying fungi on living pine are e.g. *Phaeolus schweinitzii* (Fr.) Pat., *Sparassis crispa* (Wulfen) Fr., *Phellinus pini* (Brot.) Pilát and generalistic fungi like *Armillaria* spp. and *Heterobasidion annosum* (Fr.) Bref., *Heterobasidion parviporum* Niemelä & Korhonen, *Stereum sanguinolentum* (Alb. & Schwein.) Fr., *Amylostereum areolatum* (Chaillet ex Fr.) Boidin, *Calocera furcata* (Fr.) Fr., *Trichaptum abietinum* (Pers. ex J.F. Gmel.) Ryvarden, and *Fomitopsis pinicola* (Sw.) P. Karst.

In this study, the most abundant fungus in all disease classes identified with HTS was the common foliar endophyte of Scots pine, *Sy. polyspora* [44, 89]. However, it has been noted to cause current season needle necrosis (CSNN) in true fir (*Abies* spp.) across Europe and North America [90–92], necrosis on shoots of *Pinus pinea* L. [93] as well as necrosis on stems and needles on *Pinus yunnanensis* Franch. [94]. Cleary et al. [24] suggested that this endophyte is opportunistic pathogen, that due changes in climate can potentially increase its pathogenicity. *Gremmeniella abietina* (Lagerberg) Morelet was found only from 5 samples (115 reads). The pathogen is native to Europe and produces cankers on stems and severe damages leading to the death of its main host tree species *Pinus* and *Picea* [66, 95]. Like *S. sapinea*, *G. abietina* causes crown defoliation and distortion of terminal twigs [96], leading to the assumption that it would occupies the same niches in the host tree. Due to the minor abundance of *G. abietina* in this study, it indicates to appear as an endophyte.

Role of yet unnoticed isolated yeasts

Previous studies found many yeast species in living or decaying plant parts and are often associated with other organisms like insects [71]. They are adapted to short-term fluctuations in abiotic conditions and to cyclic seasonal changes. Therefore they have physiological adaptations, like pigmentation and extracellular polysaccharides to survive [69]. Phylloplane yeasts also may influence the behavior, fitness, and growth of their hosts, because they produce plant hormone-like metabolites [69]. But there are only few taxa known to be endophytic and there are only few studies on conifer species, e.g. *Sequoia sempervirens* (D.Don) Endl. [97] *P. sylvestris* [98], and *Pinus tabuliformis* Carrière [99]. Typical endophytic plant yeasts are ascomycetous species of *Debaryomyces* Lodder & Kreger-van Rij (Saccharomycetales, Saccharomycotina), as well as basidiomycetous species of *Cryptococcus* Vuill. (Tremellales, Agaricomycotina), *Rhodotorula* F.C. Harrison, and *Sporobolomyces* Kluyver & C.B. Niel (both Sporidiobolales, Pucciniomycotina) [77, 78]. Some of the taxa were found via HTS: e.g. OTU942: *Debaryomyces* sp., OTU499: *Sporobolomyces* sp., OTU1043: *Cryptococcus* sp., or OTU802: *Rhodotorula mucilaginosa*. But only portion of the retrieved reads/OTUs could be assigned to them. Most of the identified yeast species of this study seem to have an epiphytic source. The amount of yeast taxa within shoots, twigs or stems of trees look to be low, for example Middelhoven [97] isolated only for species from young and perennial shoots of *S. sempervirens*: *Debaryomyces hansenii, Tausonia pullulans (Lindner) Xin Zhan Liu, F.Y. Bai, M. Groenew. & Boekhout* (≡ *Trichosporon pullulans* (Lindner) Diddens & Lodder) and *Trichosporon porosum(Stautz) Middelhoven, Scorzetti & Fell,* both Trichosporonales, Agaricomycotina as well as an unidentified red pigmented basidiomycetous yeast.

Beside filamentous wood decaying fungi, yeasts play an important role during the fungal transformation of wood, e.g. producing a partially de-lignified material. The efficiency in degrading of plant material differs among wood-decaying, litter-decomposing and plant-pathogenic fungi and yeasts [71]. The different decomposer groups differs in the degradation of cellulose and hemicellulose. Yeasts often form an association with basidiomycetes during the wood decay process and are able to consume lignocellulose-related sugars, usually found in tree bark, leaf litter, and rotting wood.

Mycobiome between sampling factors

Disease class

The diversity indexes and species/OTU composition were same in each disease class indicating there is no differences between the main mycobiome between different health status of pine trees (healthy versus diseased) in Diplodia tip blight-diseased forest site. Based on our data, we can hypothesize that there is no health fitness effect of mycobiome on symptomless trees. Similarly, the number of pathogens did not increase throughout the disease class. However, in a study by Martín et al [64], a correlation was found between host plant (elm) resistance to pathogens and to the structure of their microbial communities. This observation suggest that the pathogen is restricted by other mycobiome due to niche competition [2]. In our study the genotype of the trees was not defined. Instead of mycobiome, maybe the more resistant trees (defined as healthy) differ from diseased due variation in genetics of the trees.

Sampling time

As mentioned in several studies [100–102], the composition of fungal communities differs in their temporal variation due to changing weather conditions, the normal cycling of the seasons or the characteristics of the host plant. In this study, we observed statistical differences between the two sampling times (June versus September), indicating the change of mycobiome between seasons. In isolate data diversity indices were indicating higher diversity in September. However, for HTS data no differences in diversity indices were observed. *M. olivacea*, *T. conorum-piceae* and *E. nigrum* amounts increased in September based

on isolate data. Similar observations were made by Martín-Pinto et al. [103] that noted seasonal variation in fungal composition of tree seedlings (including *P. sylvestris*). Seems that the species/OTUs of pine twigs have sophisticated life strategies and species abundance is dependent of the time of the year.

Year of the growth

Compositions of the species/OTUs differed statistically between the growth years. Interestingly, the growth year 2018 had the higher diversity indices than 2017 in HTS data. Differences in diversity and frequency of isolations/species/OTUs is correlated with the length of shoots and number of tested segments (Bußkamp et al. 2020). However, the shoots of the year 2017 were often shorter than in 2018 (Table 1). This increased the statistical difference to favour of 2018. *S. polyspora* was statistically higher in HTS data in the growth year 2017 but in culture-based isolation data it was higher in growth year 2018. In needles it has observed that the frequency of the fungi increases in older needles and we were expecting this to be the case in older twigs. As the most common foliar endophyte of Scots pine, *S. polyspora* seems to be able to establish itself already in current year growth and accumulate into the older tissues.

Detection of *S. sapinea*

017. *sapinea* was the most common fungus of the isolates, but the second common fungus in the HTS analysis. If *S. sapinea* is dominant in culture-based isolation methods, it might indicate that S. sapinea is able to grow faster and/or has ability to outgrow other wood inhabiting fungi *in vitro*. This hypothetically could also happen *in planta*. *S. sapinea* was detected in healthy trees, as it was expected due to the endophytic status of the fungus. However, the abundance of *S. sapinea* was not different between different disease classes in isolate data (Fig. 8). Similarly, no difference was observed between time of sampling and year of the growth (Fig. 8). However, with HTS data the number of reads were different between sampling times, growth years and disease classes (Fig. 9). The number of *S. sapinea* reads (HTS data) were higher in June and in growth year 2017. As *S. sapinea* was more common in June, this reflects well with the production of conidial spores of this fungus. The late spring and summer months are defined as the period of proliferation [17]. However, the moisture play here a role and rainfalls favor *S. sapinea* spore dispersal regardless the time of the year [17, 34]. The abundance of *S. sapinea* was higher in older tissues, leading to the conclusion that this fungus can initiate the niche in the Scots pine woody tissue.

HTS data showed that abundance of *S. sapinea* was different as disease class 0 varied from disease classes 2, 4 and 5 (Fig. 9a). Similarly, disease class 3 was different from 2 and 4 (Fig. 9a). After removing outliers differences we detected that disease classes 2 and 4 (the number of reads) were statistically higher from disease classes 0 and 3 (Fig. 9b). Notable is, that after removing outliers, the average number of reads did not differ statistically between disease classes 0 and 5. Isolate data and HTS data (after removing outliers) both showed that the mean numbers of *S. sapinea* are similar in totally healthy trees (disease class 0, defoliation: 0-5 %) and seriously affected (disease class 5, defoliation: 81-99 %). Similarly, the isolate data could not detect differences between other disease classes. Similarly, HTS showed that disease class 2 (21-40 %) had higher abundance of *S. sapinea* compared to disease class 3 (defoliation 41-60 %). Indeed, perhaps the health status of the tree is not due the abundance of *S. sapinea* or its competition with the mycobiome. Rather the susceptibility of the tree is defined by several abiotic and biotic factors that at the moment remain still unknown. However, in an epidemiological sense, it can be assumed that the *S. sapinea* accumulate symptomless in the healthy trees. In a study by Brodde et al [34], it is discussed that the fungus can culminate for 10 years in the tree before disease outbreak might happen.

Conclusions

Environmental change is altering the disturbance regimes in many regions already [104], and the risk of disturbances for forest management is increasing under the projected climate change [48, 104, 105]. Indeed, in the current study eight trees died during the sampling period (June–September 2018) due the extreme drought conditions observed on site. We found *S. sapinea* as symptomless endophytic fungi with high abundance in healthy looking Scots pine trees. This confirms the hypothesis that *S. sapinea* is accumulating as an endophyte in the healthy trees. Similarly, this observation can explain the sudden and rapid development of disease epidemics in several areas [34, 35] and highlight the ability of the fungus to spread unnoticed [6, 23, 24]. To mitigate the possible future impacts of climate change, we need to understand all the factors that trigger the development of Diplodia tip blight disease epidemics. It is not known which abiotic or biotic factors actually activate the lifestyle switch of *S. sapinea*. This highlights the urgent need to take actions so the negative influence of *S. sapinea* is restricted and the health of pine-dominated forests is secured in changing world. The research over epidemiology of *S. sapinea – P. sylvestris* pathosystem is urgently needed. This information should be used to improve forest health (e.g. via resistance breeding) to limit the spread of pathogenic *S. sapinea*.

Methods

Sampling site

Sampling took place in June and September 2018 in a stand of Scots pine (defined as diseased due to *S. sapinea*) (52.327653 °N 11.189848 °E) close to Behnsdorf in Saxony-Anhalt, Germany. The stand consisted of pure monoculture of a single-layer mature (40-year-old) Scots pine. The area is located 150 m above sea level in a flat position in the Northwestern Harz foreland and in a moderately dry climate zone (9 °C annual mean temperature and 550 l annual mean precipitation, database: Deutscher Wetterdienst, period 1981–2010). The initial substrate is glazed loose rock over rhyolite. The soil in Behnsdorf is very acidic and low in bases, has a medium nutrient supply and is fresh in terms of water supply. The soil type was classified as brown earth and soil texture 'sand silt'. In preliminary studies the disease intensity of this stand was surveyed and classified in 6 disease classes (defoliation in percent classes 0: 0–5%, 1: >5–20%, 2: 21–40%, 3: 41–60%, 4: 61–80%, 5: 81–99%). In June 2018, 30 representative trees (five trees per disease class), were chosen for sampling (Table 1). In September 2018, eight trees sampled in June were dead, and they were replaced with new trees in the respective disease class. However, three trees could

not be replaced, and thus only 27 trees were sampled in September. Altogether we collected samples from 35 different trees, which were cut from stem height of 6–8 metres (114 branch samples, Table 1).

Three randomly selected 2-year-old twigs (growth years 2017 and 2018) per tree were collected. Two twigs were stored in 8 °C and analysed with a culture-based method within the following 48 hours. One twig per tree was immediately placed in liquid nitrogen at the site and stored in -80 °C before processed further for DNA extraction and metabarcoding. From each sampling time (June or September) samples were collected for two growth years 2017 and 2018 from each disease class (Table 1) summing up total 114 samples for HTS study and cultivation study.

Table 1
Samples collected for culture-based identification of endophytes and HTS-based identification of microbiome of Scot spine twigs collected from 35 trees com class. In respect to growth year and sampling time.

Sampling time	June												September									
Growth year	2017						2018						2017						2018			
disease class	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3
No of trees sampled	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	3	4	5	5	5	5
No of twig samples HTS	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	3	4	5	5	5	5
Amplicon sequencing successful	2	3	5	2	4	1	5	5	5	5	5	5	4	3	4	5	2	4	5	5	5	5
No of twig samples for isolation	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	6	8	10	10	10	10
No of segments incubated	47	49	51	50	43	55	56	78	72	90	77	72	54	51	55	40	23	34	58	74	63	69
No of Isolates	55	48	55	51	42	60	44	71	69	79	66	58	86	60	72	50	38	40	80	71	63	78
Mean No of Isolates per shoot	5.5	4.8	5.3	5.1	4.2	6.0	4.4	7.1	6.9	7.9	6.6	5.8	8.6	6.9	7.2	5.0	4.7	5.0	8.0	7.1	6.3	7.8
Mean No of taxa isolated	3.8	1.8	2.6	2.2	2.8	3.2	5.8	2.4	3.8	2.6	2.7	3	2.2	2.8	3	3.8	2.6	3.2	4.4	4.2	3.8	4.2

Culture-based Isolation, Morphological And Molecular Identification

The twigs were divided based on growth years, 2017 and 2018. The shoots were defoliated, washed and surface sterilized as described in Bußkamp et al. [30]. Thereafter, shoots were cut into 5 mm pieces and plated on malt yeast peptone agar (MYP) modified after Langer [106]. The Petri dishes were incubated for up to three weeks at room temperature (ca. 22 °C) at natural day/night cycle. They were visually checked for developing colonies on weekly basis. Emerging mycelia were sub-cultured separately on MYP. Isolated strains were assigned to mycelial morphotypes and identified by micromorphological characters. For identifying fungi, a ZEISS Axiostar plus microscope was used and standard procedures for fungi described in Lee and Langer [107] were followed. In addition to standard literature recommended by Oertel [108] for determination of fungi and forest diseases, the following literature was used e.g. [83, 109–117]. One representative strain of each morphotype was used for molecular identification.

Fungal DNA was extracted for molecular identification following the protocol of Keriö et al. [118]. Taq DNA polymerase (Microsynth) was used for PCR amplification of ITS regions with primer pair, ITS1-F [119] and ITS4 [120]. Briefly, the PCR protocol was as follows: 1X HR PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 100 ng template DNA, 0.2 U/µl DNA polymerase; the reaction was adjusted to 25 µl with autoclaved MQ H_2O. The PCR conditions used for ITS region were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. Possible contaminations were determined with a negative control using sterile water as template in PCR protocols. RedStain was used to confirm DNA amplicons on a 1.5% agarose gel and the visual detection was made by ultraviolet transillumination. PCR products were purified and sequenced using the ITS4 primer at Microsynth SEQLAB (Göttingen, Germany). The ITS sequences were extracted with an open source software utility (https://microbiology.se/software/itsx/) to extract the ITS2 sub-region from the fungal nuclear ITS sequences [121]. The ITS1 and ITS2 sequences were used for BLASTN [122]) searches against GenBank/NCBI [123] to

provide taxonomic identification. Intraspecific ITS similarity for the sequenced fungi of 98–100% was used at species level and further confirmed the morphological identification.

Fungal Metabarcoding And Data Analysis

The frozen twigs were divided based on growth years 2017 and 2018. The twigs were defoliated and each sample was ground using the Mixer Mill MM 400 from Retsch GmbH with a set program of 25.0 Hz for 20 s to prevent thawing of the samples. The samples and the corresponding milling equipment were handled with liquid nitrogen throughout the entire milling process. The ground product was then stored in 1.5-ml tubes at -80°C. DNA was extracted from 50 mg of the homogenized wood sample using the “innuPREP Plant DNA Kit” (Analytik Jena AG, Jena, Germany), according to the manufacturer’s instructions. DNA products were sent to Microsynth SEQLAB (Switzerland). Illumina MiSeq sequencing of amplicons were successful for 95 samples (83%) (Table 1). To sequence the internal transcribed spacer (ITS2) regions of the fungal 18S rRNA gene, two-step Nextera PCR libraries [124] using the primer pair ITS3 (5'- GCA TCG ATG AAG AAC GCA GC -3') and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3') were created [125]. Subsequently the Illumina MiSeq platform and a v2 500 cycles kit were used to sequence the PCR libraries. The produced paired-end reads which passed Illumina’s chastity filter were subject to de-multiplexing and trimming of Illumina adaptor residuals using Illumina’s real time analysis software included in the MiSeq reporter software v2.6 (no further refinement or selection). The quality of the reads was checked with the software FastQC version 0.11.8 [125]. The locus specific ITS2 primers were trimmed from the sequencing reads with the software cutadapt v2.8 [126]. Paired-end reads were discarded if the primer could not be trimmed. Trimmed forward and reverse reads of each paired-end read were merged to *in-silico* reform the sequenced molecule considering a minimum overlap of 15 bases using the software USEARCH version 11.0.667. Merged sequences were then quality filtered allowing a maximum of one expected error per merged read and discarding those containing ambiguous bases. From the remaining reads the ITS2 subregions were extracted with help of the ITSx software suite v1.1.2 [121] and its included fungi database. The extracted sequences were then denoised using the UNOISE algorithm implemented in USEARCH to form operational taxonomic units (OTUs) discarding singletons and chimeras in the process. The resulting OTU abundance table was filtered for possible bleed-in contaminations using the UNCROSS algorithm. OTUs were compared against the reference sequences of the UNITE database and taxonomies were predicted considering a minimum confidence threshold of 0.5 using the SINTAX algorithm implemented in USEARCH. Rarefaction analysis were performed with the R software packages phyloseq v1.26.1 and vegan v2.5-5. Libraries, sequencing and data analysis described in this section were performed by Microsynth AG (Balgach, Switzerland). Additional BLAST searches against NCBI genebank was done manually.

For the statistical analyses the normalized data of HTS data was used. For isolates the exact number of isolates was used. All data analyses were conducted in R version 3.5.1 [127]. For each HTS sample (95) and 114 isolate samples The Shannon-Wiener index [128] and The Simpson index [129] were calculated. The Permutational Multivariate Analysis of Variance (PERMANOVA) in VEGAN package version 2.4 [130] was used to test the statistical differences/similarities in community structure between (HTS and isolate data) samples (factors: growth year, disease class, sampling time). Permutation test (permutest.betadisper, method = bray) was used to observe and visualize the differences/similarities in dispersion between OTU composition in HTS and isolate (growth year, disease class, sampling time) in VEGAN package version 2.4 [130]. ONE-WAY-ANOVA was used to test the statistical differences/similarities in diversity indexes. *S. sapinea* reads were analysed with PERMANOVA and further with Kruskall-Wallis test (TukeyHSD test was used to search the differences between groups) for HTS data. ONE-WAY- ANOVA was used for isolate data (normally distributed) and TukeyHSD test was used to search the differences between groups (in disease classes). Welch Two Sample t-test was used for *S. sapinea* isolate data to detect differences between groups in growth year and sampling time. The statistically different OTUs (factors: growth year, disease class, sampling time) were detected with R package called ‘indicspecies’ [131].

The FUNGuild database v1.0 database (https://github.com/UMNFuN/FUNGuild) was used to assess the ecological and functional levels of OTUs identified to the species level [132]. Trophic levels included: pathogens (in FUNGuild referred to as pathotrophic fungi), saprotrophs, and mutualists (in FUNGuild referred to as symbiotrophic fungi). However, all fungal taxa were also categorized into trophic levels manually based on authors expertise and literature study. During manual curation trophic modes were assigned to endophytes, epiphytes, plant pathogens and wood-decay fungi. The plant pathogen composition between disease classes were analyzed with PERMANOVA and visualized with permutation test.

Abbreviations

HTS: high-throughput sequencing;

ITS2: internal transcribed spacer 2

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All metagenome raw data associated with this study have been submitted to the NCBI, SRA database, and can be found using accession number PRJNA645168.

Competing interests

All authors declare that they have no competing interests.

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Author Contributions

G.L., J.B., K.B, E.T. designed the experimental protocol. J.B. collected the samples in the field. J.B., K.B., E.L., conceived the experiments in the laboratory. G.L., J.B., K.B, E.T. did the manual BLAST:ing. E.T. and G.L. analyzed the results. K.B. wrote the first draft. All authors contributed to the writing of the final manuscript.

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Figures

Figure 1

Sphaeropsis sapinea - Diplodia tip blight symptoms: (a) diseased Scots pine twig with dieback of the current shoot ; (b) after seedling blight black pycnidia can be observed on the twigs; (c) affected Scots pine stand in Germany.

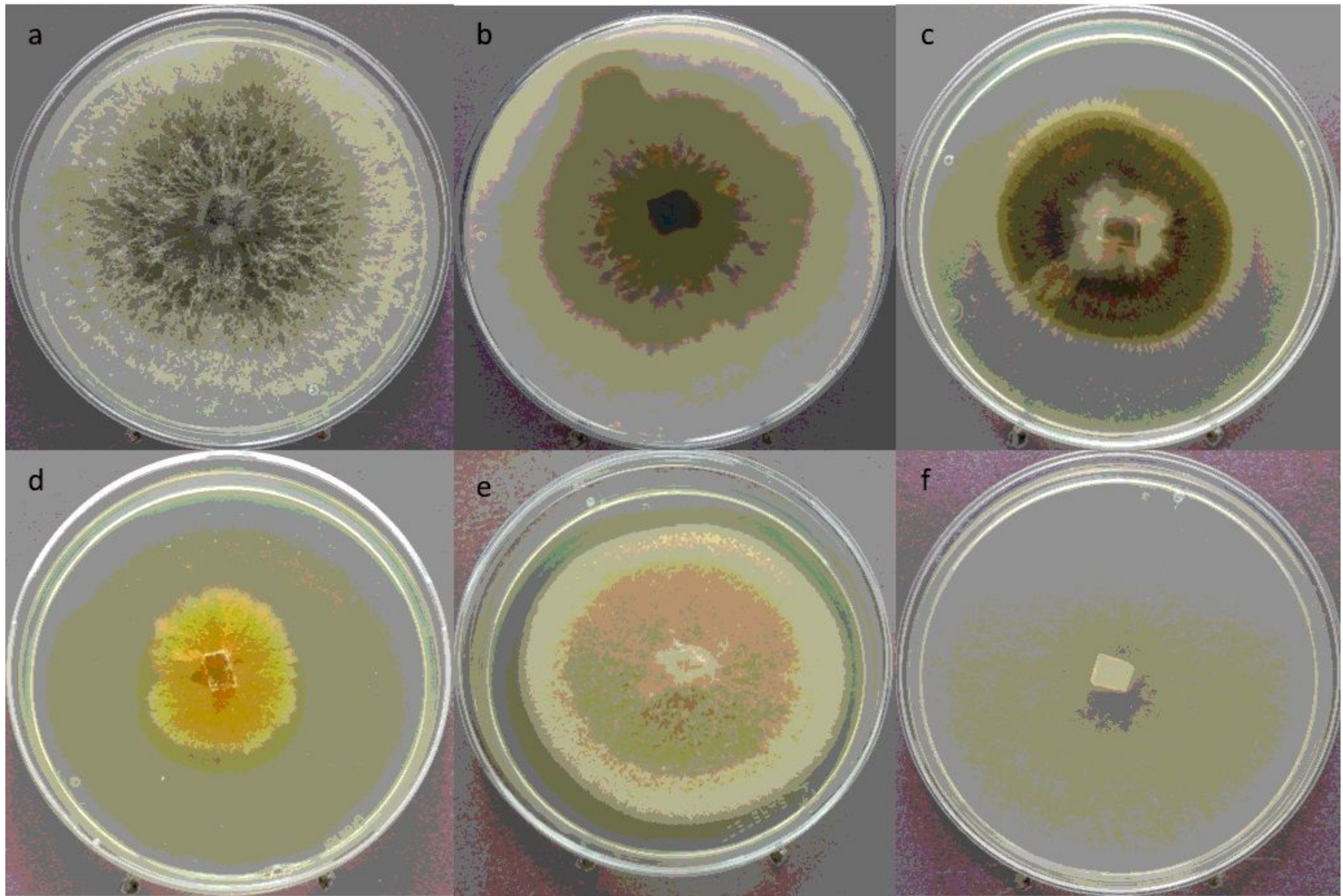

Figure 2

Most frequent isolated endophytes of Scots pine twigs, cultivated on MYP in 90 mm petridishes, 7 days in ambient daylight at room temperature, ca. 22°C: (a) Sphaeropsis sapinea obvers; (b) Sphaeropsis sapinea revers; (c) Sydowia polyspora; (d) Truncatella conorum-piceae; (e) Microsphaeropsis olivacea; (f) Desmazierella acicola

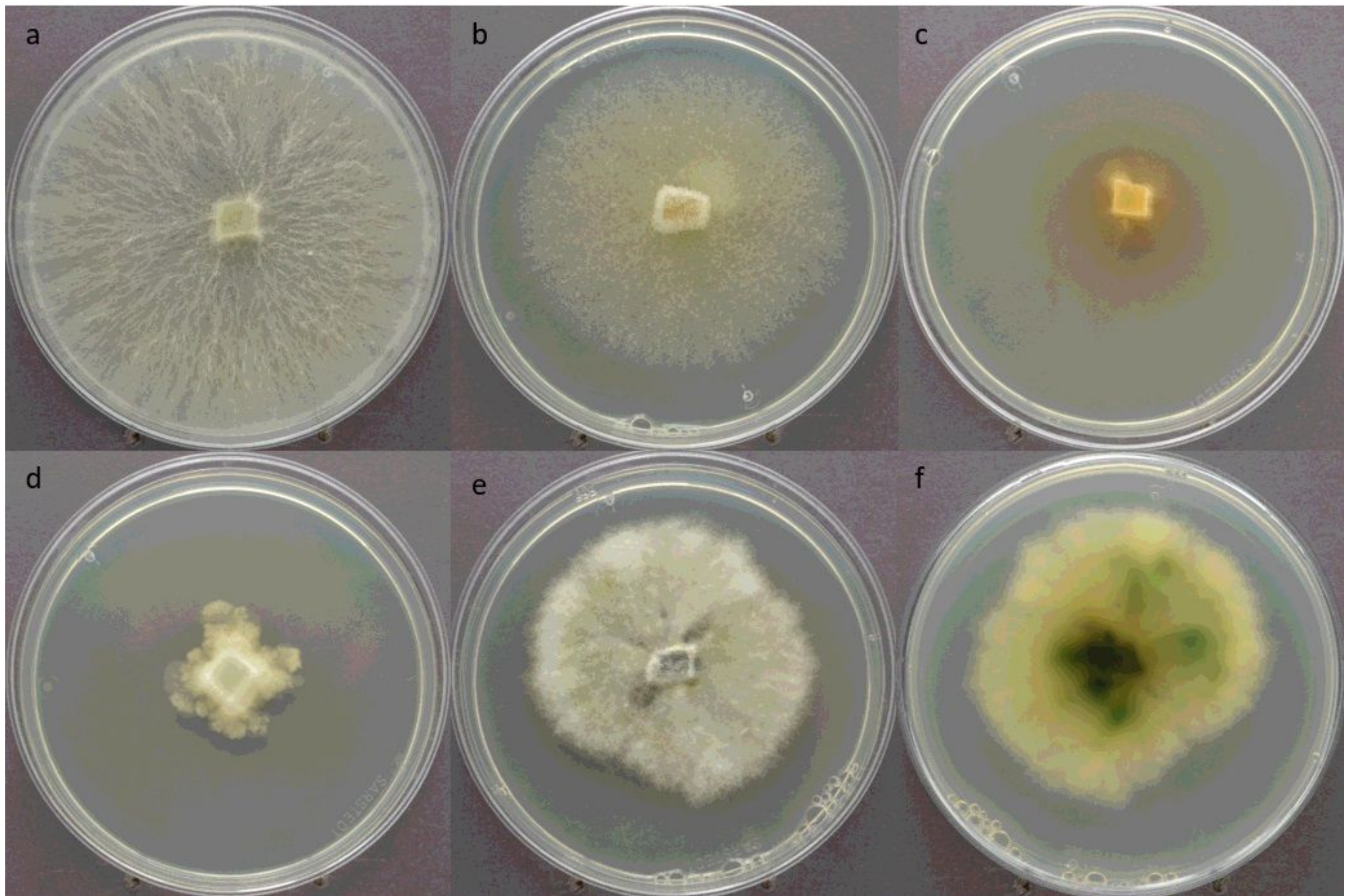

Figure 3

Endophytic Xylariales of Scots pine twigs not detected by HTS, cultivated on MYP in 90 mm petridishes, 7 days in ambient daylight at room temperature, ca. 22°C: (a-e) obvers, (f) revers; (a) Biscogniauxia mediterranea; (b) Biscogniauxia nummularia; (c) Nemania serpens; (d) Rosellinia sp.; (e-f) Hypoxylon fragiforme.

yeasts

yeasts

yeasts

Culture-based isolation method: outgrowing fungi

- yeasts
- Fungus spp. ascomycetous
- Eurotiomycetes
- Pezizomycetes
- Leotiomycetes
- Dothideomycetes
- Sordariomycetes

OTUs identified by HTS

- Basidiomycota filamentous
- Basidiomycota, divers
- Basidiomycota, Pucciniomycetes
- Basidiomycetous yeasts, yeast-like
- Ascomycetous yeasts
- Ascomycota filamentous
- Ascomycota, Lecanoromycetes
- Glomeromycota, Zygomycota, Chytridiomycota, Olpidiomycota
- Fungi sp.

Figure 4

left: Shares of outgrowing fungi obtained with cultivation method: right: Shares of OTUs identified by HTS.

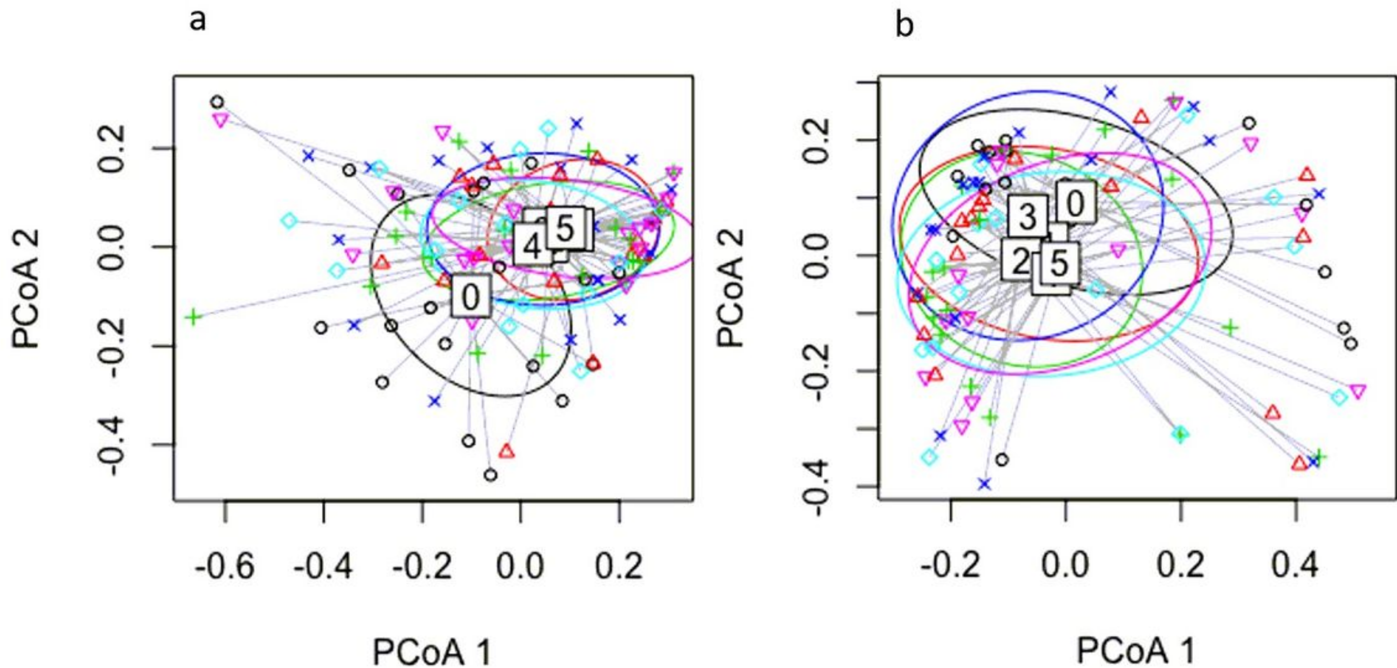

Figure 5

Dispersion of species of culture-based isolation data (a) and OTUs of HTS data; (b) of each sample (permution test with bray method) in each disease class.

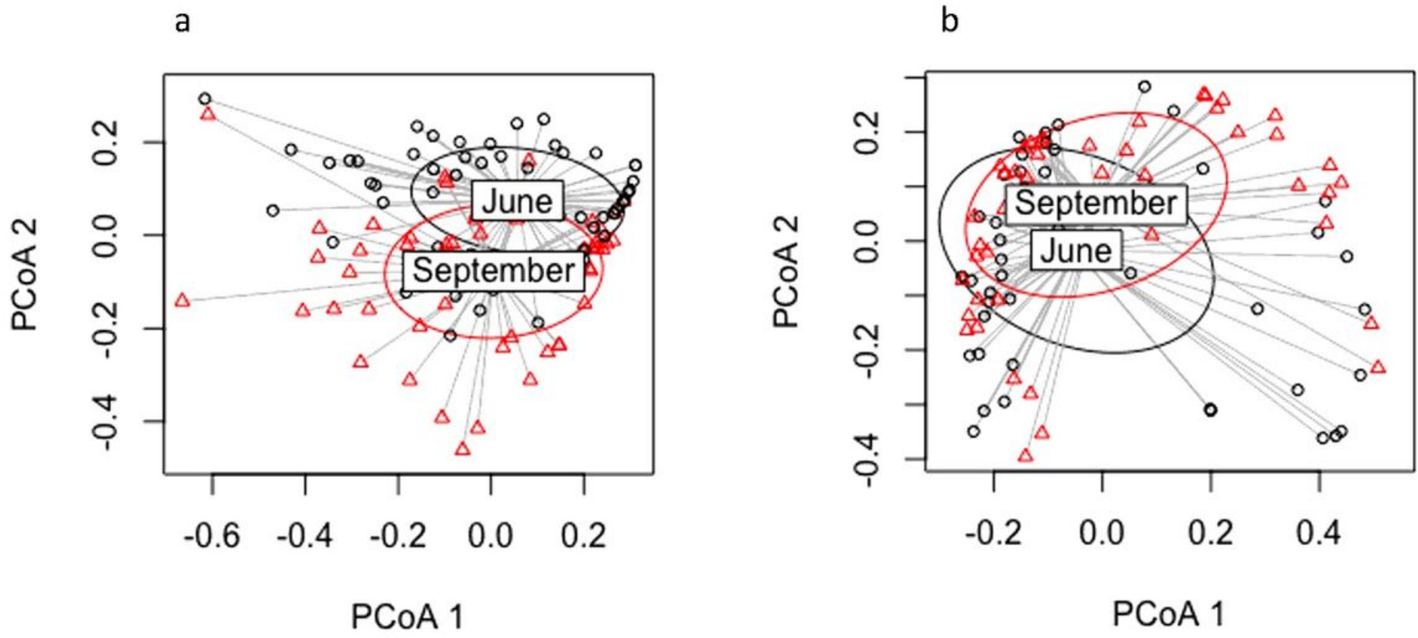

Figure 6

Dispersion of species of isolate data (a) and OTUs of HTS data; (b) in each sample between sampling times June (black) and September (red).

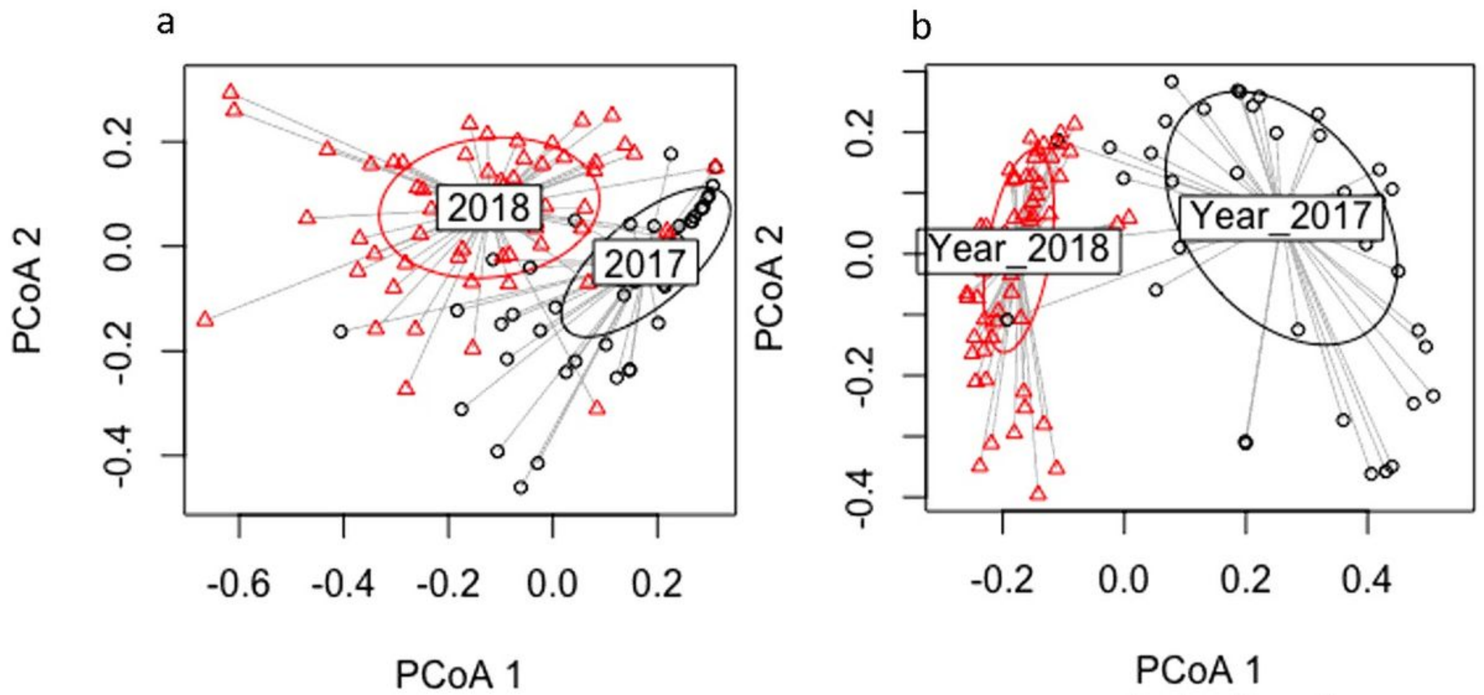

Figure 7

Dispersion of species of culture-based isolation data (a) and OTUs of HTS data; (b between growth years 2017 (black) and 2018 (red).

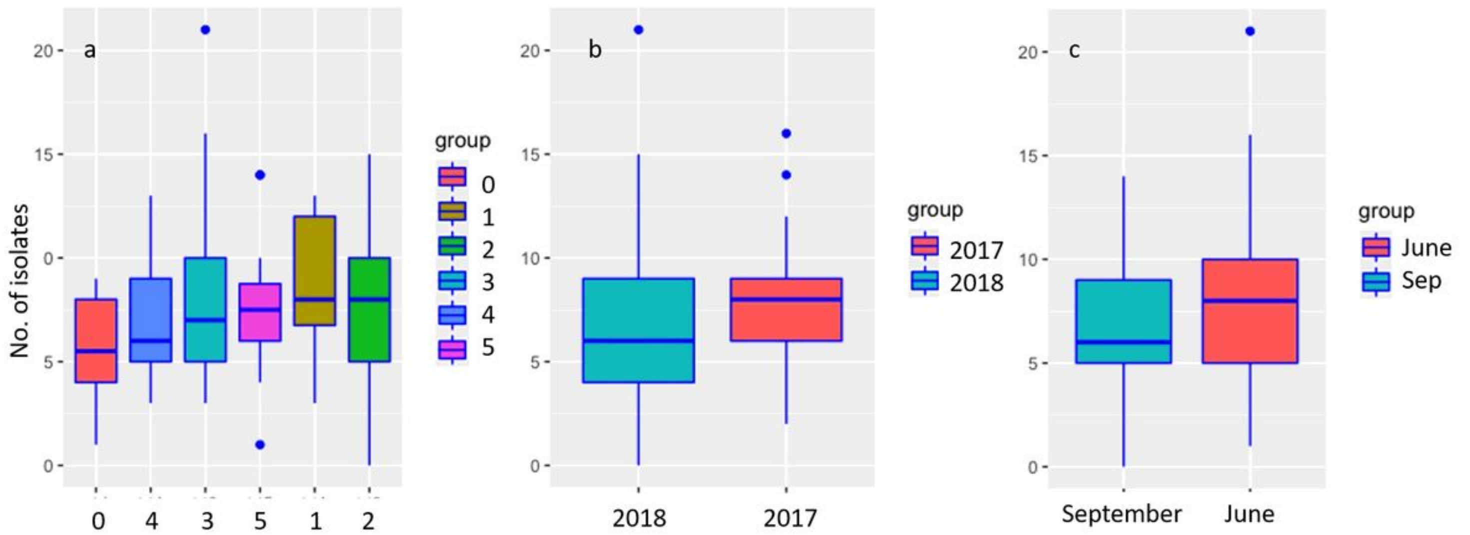

Figure 8

The number of isolations of Sphaeropsis sapinea in different disease classes (a); growth years (b); and sampling time (c) in culture-based isolation data.

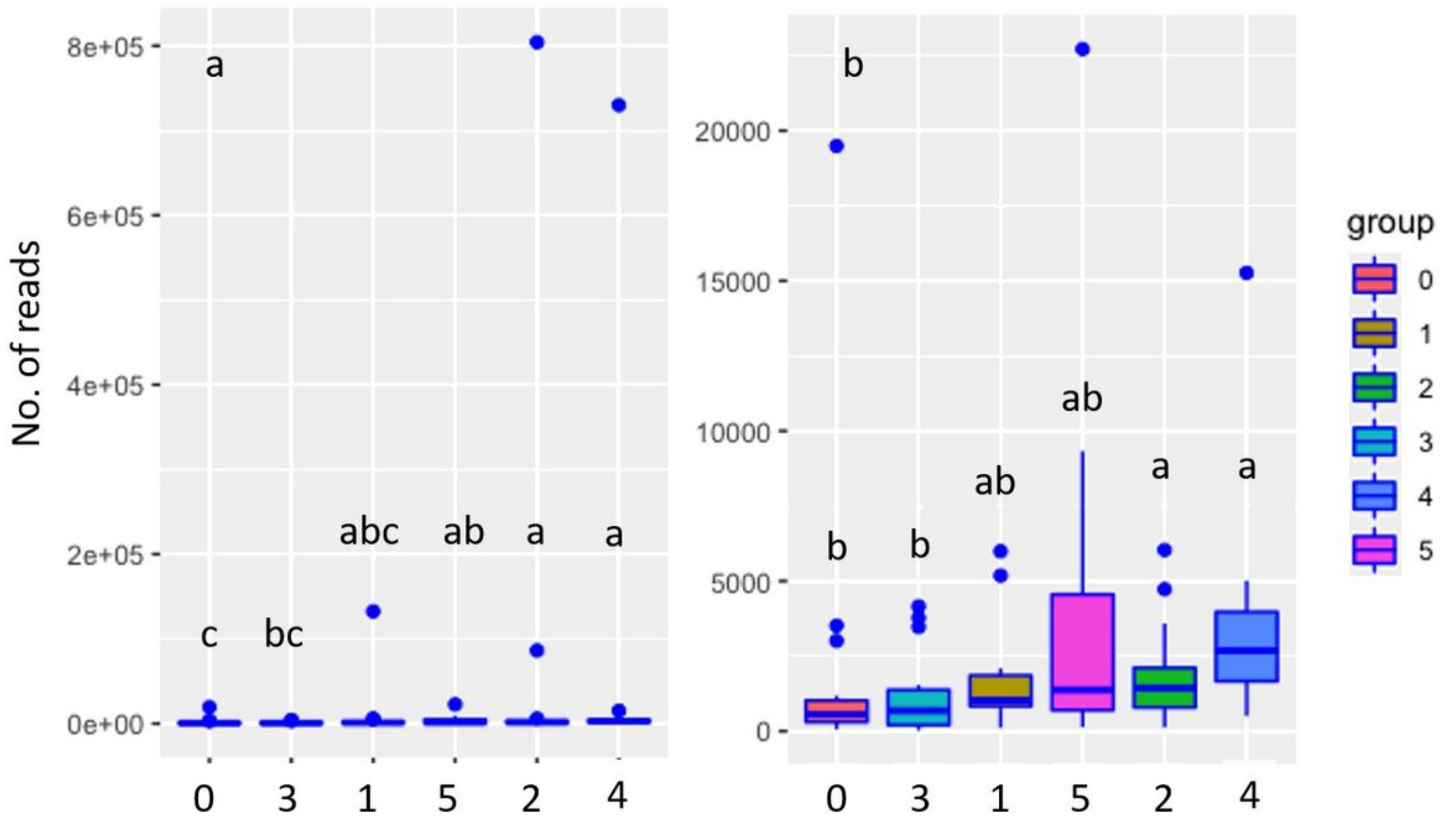

Figure 9

Boxplot of number of Sphaeropsis sapinea HTS reads observed in disease classes (0–5) with (a) and without (b) outliers.

Supplementary Files

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

- SupplementaryTable1.xlsx