Sphaeropsis sapinea and fungal endophyte diversity in twigs of Scots pine (Pinus sylvestris) in Germany

Johanna Bußkamp1,2 · Gitta Jutta Langer1 · Ewald Johannes Langer2

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Abstract

Sphaeropsis sapinea is the causal fungal agent of Diplodia tip blight disease of Scots pine (Pinus sylvestris) and other coniferous trees of relevance to forestry in Germany. In this study, the distribution and occurrence of S. sapinea and accompanying endophytic fungi in twigs of healthy and diseased Scots pine was investigated on a spatial and temporal scale. Sampling of 26,000 twig segments from trees in 105 temperate coniferous forest stands in Germany resulted in isolation of 33,000 endophytic fungi consisting of 103 species identified based on morphological and ITS-DNA sequence analyses. Approximately 98% of the sample was represented by fungi in the Ascomycota, with only two species (Peniophora pini and Coprinellus sp.) belonging to the Basidiomycota. Four species were detected in a frequency greater than 10% (Sphaeropsis sapinea, Sydowia polyspora, Microsphaeropsis olivacea, and Truncatella conorum-piceae) from the collective sample. A typical inhabitant of Scots pine twigs Desmazierella acicola was isolated and additionally typical hardwood colonizers like Biscogniauxia spp. were detected. S. sapinea, an endophytic plant pathogen with saprobic capabilities, was isolated from more than 80% of the studied pine trees, but the majority of trees sampled showed no symptoms of Diplodia tip blight. No invasive, pathogenic quarantine fungi for Germany were isolated from healthy or diseased Scots pines. Advantages and disadvantages of isolation-based endophyte studies over studies using direct DNA-isolation are discussed. Knowledge of the fungal endophyte communities in twigs of Scots pine allowed for identification S. sapinea and other potential pathogens of pines and other forest trees that may possibly contribute to increased disease under repeated periods of drought and heat stress in the future.

Keywords Endophytic fungi · Pinus sylvestris · Sphaeropsis sapinea · Diplodia tip blight

Introduction

Scots pine (Pinus sylvestris L.) is one of the most important conifer tree species in Northern and Central Europe. In Germany, this tree species is a very important factor in forest and timber management and economics. Natural Scots pine forests are rather rare in Germany, due primarily to its low competitiveness compared with other, more shade tolerant tree species such as European beech (Fagus sylvatica L.). Therefore, native pine forests are restricted to azonal vegetation types, for example, sand dunes or the edges of moors or moorlands. Most of the natural forests with Scots pine as the main tree species are found in Northeastern Germany. P. sylvestris has a wide ecological amplitude (Ellenberg and Leuschner 2010), it is considered to be a stress tolerant, easy to regenerate, versatile, and light demanding pioneer species. Typical for Northern parts of Germany are secondary or man-made pine stands. With ca. 2.4 million hectares, P. sylvestris has a 22.9% share of the German forested area (Thünen-Institut 2014); however, areas forested with pine are decreasing. This decline has several causes, but risk factors, such as global climate change and new or emerging fungal diseases, play an important role (Mason and Alía 2000; Drenkhan et al. 2016). Scots pine forests covers 28 million hectares in Europe and thus make up 20% of the commercial forest area of the European Union (Mason and Alía 2000).
**Sphaeropsis sapinea** (Fr.) Dyko & B. Sutton (syn. Diplodia pinea (Desm.) J. Kickx f.) is recognized as the most widespread necrotrophic ascomycete pathogen responsible for dramatic losses of pine trees across the continents (Fabre et al. 2011; Phillips et al. 2013). It spreads from needles via stomata or injured tissue into the host and results in disease symptoms that include tip blight, stem canker, dieback of current year shoots, and blue staining of sapwood (Brookhouser and Peterson 1971; Munck et al. 2009). It is also known to be present and persistent as a symptomless endophyte in pine twigs (Langer et al. 2011; Fabre et al. 2011; Luchi et al. 2014). Pathogenic occurrence of *S. sapinea* can be triggered by stress, which is particularly important in the context of climate change with precipitation deficits and increased temperatures that weaken pine (Fabre et al. 2011; Bosso et al. 2017). This species of the Botryosphaeriaceae family has appeared as a pathogen in Central Europe since the 1980s (Swart and Wingfield 1991). In Germany, infections of Scots pines and Austrian pines (*Pinus nigra* J.F. Arnold) by *S. sapinea* causing serious forest health problems were observed in the middle of the 1990s by Heydeck and Dahms (2012) and later by Langer et al. (2011). The geographical origin of the native distribution of *S. sapinea* is not known (CABI 2014) and it is questionable whether the species is native to Europe (Desprez-Loustau et al. 2009). Knowledge of the latent distribution of *S. sapinea* in Germany and occurrence of other potential pine pathogens or quarantine pests is of great interest. The aim of this study is to determine the composition of cultivable fungal endophytes (in the sense of Petrini (1991)) of Scots pine in Germany at the species level and to assess the taxa regarding their significance for forestry.

The distribution of *S. sapinea* in Germany, and environmental factors triggering the outbreak of disease symptoms after a period of symptomless existence of *S. sapinea* in apparently healthy twigs of pines, were studied by Bußkamp (2018) in a doctoral thesis. Results of that research are presented as part of this study. The fungal endophytes of pine branches were previously studied by Kowalski and Kehr (1992). Various other authors have studied diverse tissues of Scots pine with regard to colonization by mycobiota (Carroll et al. 1977; Petrini and Fisher 1988; Fisher et al. 1991; Kowalski and Kehr 1992; Kowalski 1993; Pirttilä et al. 2003; Lygis et al. 2004; Pinto et al. 2006; Menkis et al. 2006; Kwaśna 2008; Giordano et al. 2009; Menkis and Vasaitis 2010; Peršoh et al. 2010; Terhonen et al. 2011; Martinez-Alvarez et al. 2012; Romeralo et al. 2012; Sanz-Ros et al. 2015; Millberg et al. 2015). In contrast to the aforementioned studies, in this work the incidence of disease and endophytic occurrence of *S. sapinea* was investigated on a German spatial base with a large and comprehensive spatial and temporal sample. Additionally, an assessment of the endophytes concerning their lifestyles, trophic status, and relevance to forest health is presented.

### Materials and methods

#### Sample collection

From 2014 to 2016, in different seasons, twigs were sampled from Scots pine (*Pinus sylvestris* L.) from 105 forest sites in Germany by climbing or felling trees or by archery (Fig. 1 and supplementary Table). A systematic sampling approach was used to collect *P. sylvestris* twigs along a transect from the Northeast to the Southwest of Germany in climate sensitive regions (91 sites, Fig. 1). Transect sampling was conducted at grid points of the Forest Condition Survey grid (Thünen-Institut 2020); and 14 additional pine stands were also examined with at least 6 trees per stand being sampled (Fig. 1). Trees sampled from the latter stands were diseased by Diplodia tip blight and were also infested with phyllophagous insects (e.g., *Dendrolimus pini* L., *Thecodiplosis brachyntera* Schwägrichen) or mistletoe (*Viscum album* subsp. *austriacum* (Wiesb.) Vollm.) at the time of sampling. At the localities Pfungstadt, Bad Freienwalde, and Lüderitz, pairwise comparisons of stands with healthy and diseased trees were performed. Monthly sampling, over a period of 13 months (September 2015–October 2016), of twigs collected from a single tree was carried out in a forest stand close to the city of Dransfeld in the South of Lower Saxony (Fig. 1). Sampled trees were older than 50 years and located 20 to 600 m above sea level in stands that were dominated by Scots pine. Most stands were secondary man-made forests, partially outside the natural range of Scots pine (EUFORGEN 2008). Three shoot tips per tree were arbitrarily selected, placed in a sterile plastic bag and transported in a cool box (8 °C) to the laboratory. Twig samples were divided into two categories based on occurrence of Diplodia tip blight symptoms (healthy twigs from trees with symptoms (*n* = ~2800 samples/segments) and twigs from trees without symptoms (*n* = ~23,000 samples/segments)). Ocular visible symptoms of Diplodia dieback of shoots and browning of the crown were also recorded. Symptomatic branches were examined in the laboratory for the presence of *Sphaeropsis sapinea* (Fr.) Dyko & B. Sutton.

#### Isolation and determination of fungi

Three randomly selected tips per tree from 3 to 4-year-old branches (*n* = 190 *P. sylvestris* trees) were defoliated, washed, and surface disinfested by treating for 1 min in 70% EtOH, 5 min in a 3% NaOCl, and 1 min in 70% EtOH (Bußkamp 2018). Pre-treatments (washing and brushing), different disinfection methods (duration of soaking, concentration of NaOCl), and post-treatments (washing in water or EtOH, drying method) were tested in preliminary experiments (data not shown). The method selected for this study was verified by additional testing (imprinting, rolling in sporulating cultures of previously disinfested twigs and plating of rinse water,
followed by monitoring of fungal growth). Thereafter, twigs were cut into 5-mm length pieces (hereinafter referred to as segments), plated on malt yeast peptone agar (MYP) 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). Usually, 3 twig segments were placed on MYP medium in a 90-mm-diameter plastic Petri dish and incubated for up to 3 weeks at room temperature (ca. 22 °C) and ambient daylight. Twig segments were visually checked, weekly, for developing colonies. Emerging mycelia were sub-cultured separately on MYP medium. Isolated strains were initially assigned to mycelial morphotypes (MTs) further characterized based on micro-morphological characters and DNA sequence analysis. Representative fungal strains were stored on MYP slants at 4 °C.

From each morphotype, 1–2 mg of culture tissue was suspended in 100 μl TE buffer in a 1.5-ml tube. A microwave (600 W) was used twice for 1 min each time, with a pause of 30 s, to break up cells. Tubes were cooled to −20 °C for 20 min and centrifuged at 10000 rpm for 5 min. A 100 times diluted portion of the supernatant was used for DNA with the polymerase chain reaction (PCR). Primer pairs for amplification of the ITS1, 5.8S, and ITS2 regions were ITS1F/ITS4 or ITS1/ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR was performed with 45-μl Master mix from QIAGEN, Hilden, Germany, where 5 μl of extracted DNA was added. PCR was carried out using the primer pairs, with initial denaturation at 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 60 s; final elongation was performed at 72 °C for 7 min. PCR products were separated on 1% agarose gel stained with GelRed fluorescence dye (Biotium, Hayward, CA, USA), followed by a cleaning with QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Sanger sequencing of purified products (Sanger et al. 1977) was commissioned at GATC Biotech (Cologne, Germany). Editing and alignment of DNA sequences were performed with MEGA6 (Tamura et al. 2013) followed by submission to GenBank (Table 1).
For identifying MTs, a ZEISS Axiostar plus microscope was used and the standard procedures for fungi described in Lee and Langer (2012) were followed. In addition to standard literature recommended by Oertel (2003) for determination of fungi and forest diseases, the following literature was used, e.g., Guba 1961; Booth 1971; Domsch et al. 1993; Arx 1981; Gerlach and Nirenberg 1982; Breitenbach and Kränzlin 1984; Ju et al. 1998; Verkley 1999; Samson et al. 2010; and Butin 2011. Names of fungal species follow Index Fungorum (www.indexfungorum.org). At least one representative strain of each morphotype was used for DNA sequence analysis. Sequences were submitted to GenBank and the BLAST algorithm (http://www.ncbi.nlm.nih.gov/genbank, Altschul et al. 1997) was used for fungal taxon confirmation. Intraspecific ITS sequence similarity of 98–100% was used to determine species identity. Query coverage of all taxa was between 90 and 100%. Except for Truncatella conorum-piceae (54%) where there was only a single reference for comparison, the arithmetic mean of overlap was 97.7%. The frequency of each fungal species sampled from twigs was specified as the percentage of this particular fungus in all outgrowing fungi.

The map (Fig. 1) was made using QGIS (www.qgis.org). Evaluation of the endophyte data was analyzed using ordination (Detrended Correspondence Analysis and Canonical Correspondence Analysis, data not shown); test of significance was performed using R (R Core Team 2019, www.r-project.org).

**Results**

From 190 analyzed trees at 105 sites, 103 species of endophytic fungi were sampled (Table 1). With 101 species belonging to the Ascomycota and two species to the Basidiomycota (Peniophora pini (Schleih. ex DC.) Boidin and Coprinellus sp.). No fungi were recovered from approximately 5% of the sampled twig segments.

Fungi belonging to the Pleosporales represented the most frequent sample group of fungi (27% of the isolated algorithms) and showed a high species-richness (19 species: Alternaria alternata (Fr.) Keissl.; A. infectoria E.G. Simmons; Alternaria sp., Drechslera sp., Epicoccum nigrum Link; Microsphaeropsis olivacea (Bonord.) Höhn.; Paraphaeosphaeria neglecta Verkley, Riccioni, & Stièlow; Pa. verruculosa Verkley, Gölker, & Stièlow; Periconia sp., Phoma eurycrea Sacc., 4 unidentified Phoma species, 2 unidentified Preussia species, and 3 unidentified Pleosporaceae), whereas, e.g., the Dothideales grew out frequently (22% of the species belong to Dothideales), but only one species was detected in this order (Sydowia polyspora (Bref. & Tavel) E. Müll.). Ca. 13% of the isolated strains were assigned to Botryosphaeriales (2 species: Camarosporium brabei Marinc, M.J. Wingf. & Crous and Sphaeropsis sapinea), and respectively 10% Amphiphilae (3 species: Microdochium nivale (Fr.) Samuels & I.C. Hallett, Truncatella conorum-piceae (Tubeuf) Steyaert, and Truncatella sp. 2), 9% Xylariales (19 species: Arthrinium kogelbergense Crous, Biscogniauxia mediterranea (De Not.) Kuntze, B. nummularia (Bull.) Kuntze, Daldinia chilidiae J.D. Rogers & Y.M. Ju, D. concentrica (Bolton) Ces. & De Not., Daldinia sp., Hypoxylon fragiforme (Pers.) J. Kickx f., H. rubiginosum (Pers.) Fr., Nemania diffusa (Sowerby) Gray, N. serpens (Pers.) Gray, two unidentified Pestalotiopsis species, two unidentified Rosellinia species, Xylaria longipes Nitschke, X. polymorpha (Pers.) Grev., and three unidentified Xylaria species), 5% Sordariales (10 species: Chaetomium globosum Kunze, two unidentified Chaetomium species, Jugulospora rotula (Cooke) N. Lundq., Podospora curvicollis (G. Winter) Niesll, Sordaria fimicola (Roberge ex Desm.) Ces. & De Not., two unidentified Sordaria species, Trichocladium sp. and an unidentified Sordariales species), 5% Diaporthales (10 species: three not further identified Apioignomonia species, Cytospora sp., four unidentified Diaporthe species, Plagiostoma sp., and an unidentified Gnomoniaceae), 4% Helotiales (8 species: Botrytis cinerea Pers., Lambertella sp., Pezicula cinnamomea (DC.) Sacc., P. eurita (P. Karst.) P. Karst., P. neosporulosa Z.L. Yuan & Verkley, two unidentified Pezicula species, and Phacidium lacerum Fr.), and 2% Pezizales (5 species: Chromelosporium carneum (Pers.) Hennepert, Desmazierella acicola Lib., Peziza varia (Hedw.) Alb. & Schwein., Pyronema domesticum (Sowerby) Sacc., and an unidentified Pezizales species). The rest of the isolated species occurred with a lower abundance in the study: 6 species of the Hypocreales (Trichoderma sp., Lecanicillium psalliota (Treschew) Zare & W. Gams, Fusarium sp., Fusarium sp. 1, Fusarium solani complex, and Beauveria bassiana (Bals.-Criv.) Vuill.), Nigrospora oryzae (Berk. & Broome) Petch, Nigrospora sp., two Capnoidales species (two Cladosporium sp.), three Coniochaetales (two Lecythophora species and Coniochaeta ligniaria (Grev.) Cooke, a single Eurotiales (Penicillium sp.), a single Umbelopsidales (Umbelopsis isabellina (Oudem.) W. Gams), and few other Ascomycota not determined to the order level (Fig. 2 and Table 1).

Four fungal species were sampled with a frequency higher than 10% as follows: M. olivacea (23%), S. polyspora (22%), S. sapinea (12%), and T. conorum-piceae (10%). Fifteen species had a frequency exceeding 1%. All other species were isolated less often. S. sapinea, M. olivacea, S. polyspora, T. conorum-piceae, So. finicola, and A. alternata were isolated from more than 50% of sampled trees. The most common species were isolated from more than 80% of the studied stands along the transect: Sy. polyspora (99%), T. conorum-piceae (98%), M. olivacea (97%), and S. sapinea (88%). Other fungi that were found in more than 50% of the study areas were as follows: Diaporthe sp. 2 and A. alternata as well as Ne. serpens. Neither fungal quarantine pests (according to EPPO A1 and A2 List (EPPO 2020a, b)) nor alien species (Desprez-Loustau 2009) for Germany were identified. The following 24 species
| Taxon name                  | Frequency | % Sites | Systematics         | GenBank accession | GenBank accession | Blast            | Assessment of the significance for forestry                                                                 |
|----------------------------|-----------|---------|---------------------|-------------------|-------------------|------------------|----------------------------------------------------------------------------------------------------------------|
| Acremonium sp.             | < 0.01    | 1.9     | Hypocreales         | MG098294          | KJ194115          |                  |                                                                  |
| Alternaria alternata*      | 1.5       | 60.0    | Pleosporales        | MG098257          | KU179665          |                  | Generalist; Samson et al. (2010)                                                                                 |
| Alternaria infectoria*     | 0.3       | 30.5    | Pleosporales        | MG098290          | JQ781841          |                  | Generalist, Pathogen; Samson et al. (2010)                                                                     |
| Alternaria sp.*            | 0.1       | 3.8     | Pleosporales        | MG098289          | KJ789851          |                  |                                                                  |
| Apiognomonia sp. 3         | < 0.1     | 1.0     | Diaporthales        | MK066898          | KX776422          |                  |                                                                  |
| Apiognomonia sp. 1         | < 0.1     | 4.7     | Diaporthales        | MG098319          | EU255016          |                  |                                                                  |
| Apiognomonia sp. 2         | < 0.1     | < 1     | Diaporthales        | MG098323          | AJ884745          |                  |                                                                  |
| Arthrinium kogelbergense*  | < 0.01    | < 1     | Xylariales          | MG098297          | KF144895          |                  | Generalist, saprophyte; Crous and Groenewald (2013)                                                           |
| Ascomycete sp. 1 +         | < 0.01    | 1.9     |                    | MG098282          |                   | EF42004          |                                                                  |
| Ascomycete sp. 2 +         | < 0.1     | 2.9     |                    | MG098293          | AJ698477          |                  |                                                                  |
| Ascomycete sp. 3 +         | < 0.1     | 2.9     |                    | MG098310          | AY561220          |                  |                                                                  |
| Ascomycete sp. 4           | < 0.01    | 1.0     |                    | MK066901          | KY792589          |                  |                                                                  |
| Beauveria bassiana*        | 0.1       | 1.9     | Hypocreales         | MG098278          | KM114549          |                  | Entomopathogen; Brady (1979)                                                                                    |
| Biscogniauxia mediterranea*| 1.8       | 48.6    | Xylariales          | MG098274          | KT823762          |                  | Pathogen, endophyte, typical hard wood colonizer; Jong and Rogers (1972); Breitenbach and Kränzlin (1984); Henriques et al. (2014a, b) |
| Biscogniauxia mummularia*  | 0.7       | 39.0    | Xylariales          | MG098283          | EF155488          |                  | Typical hard wood colonizer, endophyte, weakness pathogen, saprophyte; Fournier and Magni (2004a); Luchi et al. (2006) |
| Botrytis cinerea*          | 0.1       | 5.7     | Helotiales          | MG098288          | CP009808          |                  | Pathogen; Domsch et al. (1993); Samson et al. (2010)                                                          |
| Camarosporium brabeji*     | 0.5       | 30.5    | Botryosphaeriales   | MG098280          | EU552105          |                  | Pathogen and saprophyte; Botella and Diez (2011)                                                               |
| Chaetomium globosum*       | 0.1       | 10.5    | Sordariales         | MG098250          | FN868476          |                  | Generalist; Minter (2006); Samson et al. (2010)                                                               |
| Chaetomium sp. 1*          | 0.3       | 12.4    | Sordariales         | MG098335          | FJ666356          |                  |                                                                  |
| Chaetomium sp. 2*          | < 0.1     | 4.8     | Sordariales         | MG098270          | KT371334          |                  |                                                                  |
| Chromelosporium carneum*   | < 0.01    | 1.9     | Pezizales           | MG098273          | FJ872075          |                  | Saprophyte; Hennebert (1973)                                                                                    |
| Cladosporium sp. 1         | < 0.01    | < 1     | Capnodiales         | MG098291          | KM520367          |                  |                                                                  |
| Cladosporium sp. 2         | < 0.01    | 1.0     | Capnodiales         | MG098295          | HF952649          |                  |                                                                  |
| Coniochaeta ligniaria*     | < 0.1     | 8.6     | Coniochaetales      | MG098287          | AY198390          |                  | Generalist; Weber (2002)                                                                                        |
| Cytospora sp.              | < 0.01    | 1.0     | Diaporthales        | MK066904          | KY051969          |                  |                                                                  |
| Daldinia chilindae*        | 0.4       | 6.7     | Xylariales          | MG098286          | KJ957789          |                  |                                                                  |
| Daldinia concentrica*      | 0.2       | 18.1    | Xylariales          | MG098285          | AM292046          |                  | Typical hard wood colonizer, saprophyte; Stadler et al. (2014)                                                  |
| Daldinia sp.               | < 0.01    | < 1     | Xylariales          | MK066899          | AM292038          |                  | Typical hard wood colonizer, saprophyte; Breitenbach and Kränzlin (1984); Stadler et al. (2014)                  |
| Desmazierella acicola*     | 2.1       | 43.8    | Pezizales           | MG098266          | LN589957          |                  |                                                                  |
| Taxon name                        | Frequency | % Sites | Systematics | GenBank accession | GenBank accession Blast | Assessment of the significance for forestry |
|----------------------------------|-----------|---------|-------------|-------------------|-------------------------|-----------------------------------------------|
| Typical saprophyte of *P. sylvestris* needles; Przybył et al. (2008); Martinović et al. (2016) |           |         |             |                   |                          |                                               |
| Diaporthe sp. 1*                 | 0.4       | 24.8    | Diaporthales | MG098256          | KC145855                 |                                               |
| Diaporthe sp. 2*                 | 3.2       | 60.0    | Diaporthales | MG098258          | EF155490                 |                                               |
| Diaporthe sp. 3                  | 0.2       | 15.2    | Diaporthales | MG098269          | KJ609006                 |                                               |
| Diaporthe sp. 4*                 | 0.6       | 2.8     | Diaporthales | MG098318          | KC343205                 |                                               |
| Drechslera sp.                   | 0.1       | 2.9     | Pleosporales | MG098259          | GU067763                 |                                               |
| Epicoccum nigrum*               | 1.3       | 47.6    | Pleosporales | MG098251          | GU566259                 | Generalist; Fávaro et al. (2011); Samson et al. (2010) |
| Fusarium solani complex          | <0.01     | <1      | Hypocreales  | MK066900          | MF327377                 | Potential pathogen; Butin (2011)              |
| Fusarium sp. 1*                 | 0.3       | 23.8    | Hypocreales  | MG098263          | HQ630964                 |                                               |
| Fusarium sp. 2                  | <0.01     | <1      | Hypocreales  | MK066908          | AF310980                 |                                               |
| Gnomoniaceae sp.                | 0.5       | 39.0    | Diaporthales | MG098311          | DQ872667                 |                                               |
| Hypoxylon fragiforme*            | 1.0       | 28.6    | Xylariales   | MG098276          | E55528                   | Typical hard wood colonizer, saprophyte; Greenhalgh and Chesters (1968); Breitenbach and Kränzlin (1984); Rogers et al. (2002) |
| Hypoxylon rubiginosum*           | <0.01     | <1      | Xylariales   | MG098300          | KO96929                  | Typical hard wood colonizer, saprophyte; Breitenbach and Kränzlin (1984); Rogers et al. (2002) |
| Jugulosphora rotula*            | <0.01     | <1      | Sordariales  | MG098304          | AY999136                 | Saprophyte; Lundqvist (1972)                   |
| Lambertella sp.                 | <0.1      | 1.0     | Helotiales   | MG098309          | KF499362                 | Entomopathogen; generalist; Zare and Games (2003); Arevalo et al. (2009) |
| Lecaniiarium psalliota*+         | 0.1       | 3.8     | Hypocreales  | MG098334          | AY261180                 |                                               |
| Lecythophora sp. 1*             | 0.0       | 1.9     | Coniochaetales | MG098271          | KF367565                 |                                               |
| Lecythophora sp. 2*             | 0.1       | 10.5    | Coniochaetales | MG098277          | KT264687                 |                                               |
| Leotiomycetes sp.               | <0.1      | <1      |              | MG098324          | KC595271                 |                                               |
| Microdochium niveale*            | <0.1      | 1.9     | Amphiphaeriales | MG098306          | AM502166                 | Generalist; Liu et al. (2015); Chen et al. (2015) |
| Microsphaeropsis olivacea*       | 23.0      | 97.1    | Pleosporales | MG098249          | JX681101                 | Typical hard wood colonizer, saprophyte; Fournier and Magni (2004b); Balasuriya and Adikaram (2009) |
| Nemania diffusa*+               | 0.7       | 35.2    | Xylariales   | MG098265          | KT23181                  | Typical hard wood colonizer; Breitenbach and Kränzlin (1984) |
| Nemania serpens*                | 1.5       | 50.5    | Xylariales   | MG098299          | E555504                  | Typical hard wood colonizer; Breitenbach and Kränzlin (1984) |
| Nigrospora oryzae*              | 1.3       | 15.2    | Incertae sedis | MG098254          | EU436680                 | Generalist; Sivanesan and Holiday (1971)      |
| Nigrospora sp.                  | <0.1      | 1.9     | Incertae sedis | MG098279          | KC505176                 | Generalist; Verkley et al. (2014)             |
| Paraphaeosphaeria neglecta*     | <0.01      | <1     | Pleosporales | MG098298          | JX496092                 | Generalist; Verkley et al. (2014)             |
| Paraphaeosphaeria verruculosa*   | <0.1      | 2.9     | Pleosporales | MG098314          | JX496059                 | Generalist; Verkley et al. (2014)             |
| Penicillium sp.                 | <0.01     | <1     | Eurotiales | MG098313          | KF367536                 |                                               |
| Periconia sp.                   | <0.01     | <1     | Pleosporales | MG098330          | JX981482                 |                                               |
| Taxon name          | Frequency  | % Sites | Systematics | GenBank accession | GenBank accession | Blast  | Assessment of the significance for forestry |
|---------------------|------------|--------|-------------|-------------------|-------------------|--------|---------------------------------------------|
| Pestalotiopsis sp. 1 | < 0.1      | < 1    | Xylariales  | MG098316          | KM129328          |        |                                             |
| Pestalotiopsis sp. 2 | < 0.01     | < 1    | Xylariales  | MG098325          | EUS2147           |        |                                             |
| Pezicula cinnamomea | 0.7        | < 1    | Helotiales  | MG098321          | KR859162          |        | Typical hard wood colonizer, Pathogen; Verkley (1999) |
| Pezicula euoria*    | 0.9        | 26.7   | Helotiales  | MG098307          | KR859188          |        | Saprophyte; Verkley (1999)                  |
| Pezicula neosporulosa* | 0.6      | < 1    | Helotiales  | MG098320          | KR859231          |        | Pathogen; Yuan and Verkley (2015)          |
| Pezicula sp. 1      | 1.4        | < 1    | Helotiales  | MG098317          | KR859220          |        |                                             |
| Pezicula sp. 2      | 0.3        | < 1    | Helotiales  | MG098322          | AF141166          |        |                                             |
| Peziza varia*       | < 0.01     | < 1    | Pezizales   | MG098331          | FN868472          |        | Saprophyte; Breitenbach and Kränzlin (1984) |
| Pezizales sp.       | < 0.01     | < 1    | Pezizales   | MK066903          | MG762599          |        |                                             |
| Pezizomyces sp. 3   | < 0.01     | < 1    | Pezizales   | MK068897          | JQ758844          |        |                                             |
| Pezizomyces sp. 1*  | 0.2        | 16.2   | Pezizales   | MG098267          | Q1538018          |        |                                             |
| Pezizomyces sp. 2   | < 0.01     | < 1    | Pezizales   | MG098332          | FN868473          |        |                                             |
| Phacidium lacerum   | 0.2        | 13.3   | Helotiales  | MG098312          | KU942438          |        | Typical saprophyte of P. sylvestris needles; Nawrot-Chorabik et al. (2013, 2016); Crous et al. (2014) |
| Phoma eupyrena*     | < 0.1      | 5.7    | Pleosporales| MG098275          | AJ890436          |        | Pathogen; Hampel (1970); Domsch et al. (1993); Hansen et al. (2013) |
| Phoma sp. 1         | < 0.01     | < 1    | Pleosporales| MG098301          | JX421725          |        |                                             |
| Phoma sp. 2         | < 0.01     | < 1    | Pleosporales| MG098303          | JQ318008          |        |                                             |
| Phoma sp. 3         | < 0.01     | < 1    | Pleosporales| MG098328          | EU16565           |        |                                             |
| Phoma sp. 4         | < 0.01     | < 1    | Pleosporales| MK066907          | JX160059          |        |                                             |
| Phlogiostoma sp.    | < 0.01     | < 1    | Diaporthales| MK066906          | MH864035          |        |                                             |
| Pleosporaceae sp.   | 0.1        | 1.0    | Pleosporales| MG098327          | KT268913          |        |                                             |
| Pleosporales sp. 1  | < 0.01     | 1.0    | Pleosporales| MK068896          | JF288444          |        |                                             |
| Pleosporales sp. 2  | < 0.01     | < 1    | Pleosporales| MK06905           | KT268865          |        |                                             |
| Podospora curvillo* | < 0.1      | 1.0    | Sordariales | MG098296          | G0225462          |        | Saprophyte; Mirza and Cain (1969); Lundqvist (1972) |
| Prussia sp. 1       | < 0.1      | 1.0    | Pleosporales| MG098305          | F120518           |        |                                             |
| Prussia sp. 2       | 0.1        | < 1    | Pleosporales| MG098329          | JN225886          |        |                                             |
| Pyronema domesticum* | 0.3   | 14.3   | Pezizales   | MG098284          | HQ115722          |        | Buring areas; Breitenbach and Kränzlin (1984) |
| Rosellinia sp. 1    | < 0.01     | 1.0    | Xylariales  | MG098260          | FN435734          |        |                                             |
| Rosellinia sp. 2+   | 1.1        | 38.1   | Xylariales  | MG098268          | KC311485          |        |                                             |
| Sordaria fimicol*a  | 4.8        | 22.9   | Sordariales | MG098253          | FN868475          |        | Generalist; Lundqvist (1972)                |
| Sordaria sp. 1*     | 0.1        | 8.6    | Sordariales | MG098252          | JN207268          |        |                                             |
| Sordaria sp. 2      | < 0.01     | < 1    | Sordariales | MK06902           | KY742564          |        |                                             |
| Sordariales sp.     | < 0.01     | 1.9    | Sordariales | MG098264          | HY999926          |        |                                             |
| Sphaeropsis sapinea* | 12.2  | 87.6   | Botryosphaeriales| MG098333  | KF766159          |        |                                             |
| Taxon name                        | Frequency | % Sites | Systematics       | GenBank accession | GenBank accession | Blast Accession | Assessment of the significance for forestry                                                                 |
|----------------------------------|-----------|---------|-------------------|-------------------|-------------------|-----------------|----------------------------------------------------------------------------------------------------------------|
| *Sydovia polyspora*              | 22.1      | 99.0    | Dothideales       | MG098248          |                  |                 | Pathogen, saprophyte, typical endophyte of *P. sylvestris*; Swart and Wingfield (1991)                           |
| *Sydovia polyspora*              | <0.1      | 3.8     | Sordariales       | MG098272          |                  |                 | Typical Endophyte of *P. sylvestris* twigs, potential pathogen; Sutton and Waterston (1970); Brener et al. (1974); Heydeck (1991); Talgø et al. (2010); Heydeck and Dahms (2012) |
| *Trichocladium* sp.              | <0.1      | 6.7     | Hypocreales       | MG098255          |                  |                 | Weakness pathogen, saprophyte; Guba (1961); Maharachchikum-bura et al. (2011); Landeskompetenzzentrum Forst Eberswalde (2016) |
| *Truncatella conorum-piceae*     | 10.4      | 98.1    | Amphisphaeriales  | MG098255          |                  |                 | Generalist; Meyer and Gams (2003)                                                                             |
| *Truncatella* sp. 1              | <0.1      | <1      | Amphisphaeriales  | MG098315          |                  |                 | Typical hard wood colonizer; Breitenbach and Kränzlin (1984)                                                |
| *Umbelopsis isabellina*          | <0.1      | 1.9     | Umbelopsidales    | MG098308          |                  |                 | Typical hard wood colonizer, saprophyte; Breitenbach and Kränzlin (1984)                                   |
| *Xylaria longipes*               | 0.8       | 42.9    | Xylariales        | MG098261          |                  |                 | Typical saprophyte of *P. sylvestris*; Bernicchia and Gorjón (2010)                                        |
| *Xylaria polymorpha*             | 0.1       | 5.7     | Xylariales        | MG098262          |                  |                 |                                                                                                              |
| *Xylaria* sp. 1                  | 0.1       | 12.4    | Xylariales        | MG098281          |                  |                 |                                                                                                              |
| *Xylaria* sp. 2                  | <0.01     | <1      | Xylariales        | MG098292          |                  |                 |                                                                                                              |
| *Xylaria* sp. 3                  | <0.1      | 1.9     | Xylariales        | MG098302          |                  |                 |                                                                                                              |
| *Coprinellus* sp.                | 0.1       | 1.0     | Agaricales        | MG098326          |                  |                 |                                                                                                              |
| *Peniophora pini*                | <0.01     | <1      | Russulales        | MG547963          |                  |                 |                                                                                                              |
were found as endophytes in pine twig for the first time: Ar. kogelbergense, B. nummularia, Bo. cinerea, Ca. brabeji, Ch. globosum, Co. ligniaria, Chr. carneum, Da. childiae, Hy. rubiginosum, Ju. rotula, Le. psalliotae, Mi. nivele, Ne. diffusa, Pa. neglecta, Pa. verruculosa, Pen. pini, Pez. neosporulosa, Pez. varia, Ph. eupyrena, Py. domesticum, Po. curvicolla, T. conorum-piceae, U. isabellina, and X. polymorpha (Table 1, species denoted with a plus # symbol).

Along the transect, 5–22 endophyte species per tree were detected, on average 13 species per plot. The number of identified species per plot increased with the number of investigated samples. Typically three pine twigs were sampled per tree (between 22 and 213 studied segments per tree). But a single tree located in the sampling area “Dransfeld” was sampled monthly over 13 months (~7500 studied segments) and 84 species were isolated. Seasonal differences in the frequency and number of species were apparent. For this tree, the colonization rate (number of species and frequency) was lower in December to March than from April to November. The occurrences of Sy. polyspora and M. olivacea contrasted, with Sy. polyspora dominating in the summer months (June–July) and M. olivacea in December–March.

At least one S. sapinea-strain was sampled from 88% of the 105 studied pine stands. In forest stands without symptoms of Diplodia tip blight, S. sapinea was detected with a relative frequency ranging from 0 to 68%. The analysis of the endophytic occurrence of S. sapinea along the studied transect across Germany exhibited that geographic longitude and altitude do not significantly influence the occurrence of the dieback fungus. However, S. sapinea tended to be isolated in a lower frequency at sites with higher altitudes (Pearson correlation coefficient = −0.29).

From diseased tree twig samples, 45 fungal species were isolated (See Table 1, species denoted with a plus + symbol).

All species of endophytic fungi found in diseased trees could also be isolated from trees without symptoms. A pairwise comparison of sites with and without symptoms of Diplodia tip blight revealed that the number of endophytic species isolated in trees with and without symptoms at sites in Pfungstadt and Bad Freienwalde were similar (Pfungstadt 34 and 33 species respectively, Bad Freienwalde in each case 23 species), with the occurrence of the different species also varying little between symptomatic and symptom-free trees in intensively examined stands. However, differences were found between healthy and diseased trees with regard to the infection rate with S. sapinea: 9% in healthy trees and 40% in diseased trees. In all three comparative pairs (Pfungstadt, Bad Freienwalde, and Lüderitz), the frequency of occurrence of S. polyspora and Desmazierella acicola were higher in healthy trees.

Twelve isolated species were identified as potential pathogens on woody plants as follows: A. infectoria, B. mediterranea, B. nummularia, Bo. cinerea, Ca. brabeji, Fusarium solani complex, Pez. cinnamomea, Pez. neosporulosa, Ph. eupyrena, S. sapinea, Sy. polyspora, and T. conorum-piceae (Table 1).

Discussion

The comparison of the endophyte fungal twig communities of diseased and non-diseased Scots pine trees revealed few differences. Except S. sapinea, there were no isolated species which occurred or were specific to diseased Scots pines. Botella et al. (2010) detected Sy. polyspora in twigs of diseased Aleppo pine. Additionally, several other potentially pathogenic fungi, e.g., Gremmeniella abietina (Lagerb.) M. Morelet, Cytospora sp., Naemacyclus niveus (Pers.) Fückel ex Sacc., and Pestalotiopsis stevensonii Peck were observed by Botella et al., but these pathogens were not found in the tested Scots pine twigs in the present
study. Sy. polyspora was isolated on nearly every studied site (99%) in this study. On the one hand, it was classified as a typical endophyte because no pathogenic occurrence (no typical disease symptoms) was observed in all tested trees. On the other hand, it could be assessed as a potential pathogen for (coniferous) trees based on various published studies (Table 1).

It was apparent that pines with Diplodia tip blight studied in Germany exhibited a very high rate of infection with S. sapinea (~40%). Although high rates of infection were rarely, also found in symptomless and vital pine stands (e.g., 68% S. sapinea at a sampling point in Hesse), the average rate of infection of symptomless pines was lower (~9%). The number of species isolated from diseased trees was generally less (n = 45, see Table 1) than those isolated out of symptomless pines (n = 103). This may be partially explained by the fast growth of S. sapinea in culture (Slippers and Wingfield 2007; Decourcelle et al. 2015), which possibly resulted in an underrepresentation of slow-growing fungal species. The sample numbers for the two categories (symptomless and Diplodia-diseased pines) in this study differed (2800 tested twig segments of pine twigs from trees with symptoms or 23,000 without symptoms of the Diplodia tip blight). This was probably the most significant influence on the detected number of species. In this study, S. sapinea was isolated from twigs with a relative frequency of 12% (mean value for all examined twig segments). From 88% of the 105 examination sites, it was possible to isolate at least one S. sapinea strain. The endophytic occurrence of S. sapinea in this study, measured in frequency of colonization, is higher than in other studies like by Bihon et al. (2011), Flowers et al. (2001, 2003), and Maresi et al. (2007). Bihon et al. (2011) assumed that the low isolation frequency of endophytic S. sapinea could be explained by the specific position of its propagules in symptomless pine tissue. The colonization with S. sapinea in buds and bark of black pine is not continuous, as presented in the study of Flowers et al. (2003). In their experiment, they bisected buds and bark and could not always isolate S. sapinea from both parts. Maresi et al. (2007) detected more endophytic growing S. sapinea strains with the PCR-method, compared with using isolation on nutrient media, from twigs of black pine. There are S. sapinea selective media, such as Swart’s medium (Swart et al. 1987) or Blodgett’s medium (Blodgett et al. 2003). Rigling et al. (1989) described the highest frequency of isolation on Bavendamm’s medium. In our opinion, the use of a selective medium is not always necessary, since S. sapinea grows well, fast, and competitive on the used MYP medium.

On sites with a higher altitude, S. sapinea tended to be isolated with a lower frequency. This tendency was first described by Fabre et al. (2011) who also found a decrease in S. sapinea colonization of pine cones with increasing elevation. These researchers assume, therefore, that the milder temperature in winter at sites with lower elevation could be an explanation. In this study, the range in height of the examined sites was between ~20 and 600 m above sea level, while the researcher group around Fabre et al. (2011) examined sites up to ~1500 m above sea level.

The widespread latent/endophytic infection of pines with S. sapinea constitutes a danger to weakened pine stands. It is presumed that S. sapinea already present in a pine can easily change from endophytic to parasitic lifestyle in a weakened host. In greenhouse experiments, Stanosz et al. (1997) and Flowers et al. (2001) proved that S. sapinea strains obtained from healthy pine tissues show an equally high pathogenic potential as those strains obtained from diseased tissues. Usually numerous pycnidia of S. sapinea are present in forest stands occurring on twigs, cones, and needles of pine. They represent a constant risk for infection due to airborne dispersal of conidia. A high latent infection rate may pose a large risk when there are disease-triggering factors, e.g., hail or insect feeding or extreme weather conditions such as heat and drought, as in the years 2018 und 2019 in Germany.

The question of whether S. sapinea is native in Germany could not be answered with the presented results but the wide endophytic distribution suggests that this is possible.

Sydowiacytomyces polyspora was the fungus with the highest abundance in this study. Twenty-two percent of all isolates were identified as Sy. polyspora and it was detected on nearly all sites (99%). Additionally, its infection rate in healthy trees was much higher than in the studied diseased pines. This is in agreement with the results of other studies, which demonstrate that Sy. polyspora occurs often as an epiphyte or endophyte of conifers and is widely distributed around the world (Muñoz-Adalia et al. 2017; Pan et al. 2018). It was found to be the second most common fungus in twig tissues of P. sylvestris (Sanz-Ros et al. 2015). Sy. polyspora lives predominantly saprophytically on a dead plant material and occurs on previously damaged needles and twigs as a weak pathogen (Heydeck 1991). Ascomata and pycnidia appear on dead pine branches and needles (Gremmen 1977). Sy. polyspora is also a wound pathogen and a blue stain fungus (Sutton and Waterston 1970). This is contrary to observations that this species causes damage in other conifers (Butin 1964), e.g., current season needle necrosis (CSNN) on true fir (Talgø et al. 2010), distinct chlorosis, or discoloration of needles and phloem lesions in Pinus yunnanensis Franchet (Pan et al. 2018). Therefore, it seems to be a potential pathogen for the studied Scots pine trees.

Microosphaeropsis olivacea (basionym: Coniothyrium olivaceum Bonord.) was isolated from nearly all studied stands (97%) and it was the third most common fungus in this study, with an abundance of 23%. Surprisingly, M. olivacea is only mentioned in three other studies as an endophyte of pine (Petrini and Fisher 1988; Kowalski and Kehr 1992; Kowalski 1993). It is also an endophyte in other tree species (e.g., Hormazabal et al. 2005). Anamorphic, Coniothyrium-like fungi are often colonizers of wood and leaves of woody plants (Damm et al. 2008). M. olivacea is known to be plurivorous and was found on twigs and branches of Cytisus, Hedera, Laurus, Lycium, and Sambucus (Ellis and Ellis 1985). Recently it was found to be the causal agent of brown spine
rot of Camelthorn (Alhagi maurorum Medik.) (Razaghi and Zafari 2016). Additionally, it was identified as an etiological agent of human skin infection (Guarro et al. 1999).

Truncatella conorum-piceae (= Pestalotia conorum-piceae Tubeuf) was also a frequently isolated endophyte in this study (frequency 10%, isolated on 98% of all sites). This was unexpected, because in other studies on endophytes in twigs of *P. sylvestris* no fungi allied to Truncatella s. l. were mentioned (Petriini and Fisher 1988; Kowalski and Kehr 1992; Persöhn et al. 2010; Martínez-Álvarez et al. 2012; Sanz-Ros et al. 2015). This fungus is mainly a saprobe and is only known as a subsequent decomposer of pre-damaged needles of pine (Landeskompetenzzentrum Forst Eberswalde (LFE) 2016). In other tissues of Scots pine Truncatella angustata, respectively, Truncatella spp. were rarely isolated (Menkis et al. 2006; Menkis and Vasaitis 2010; Terhonen et al. 2011).

Desmazerella acicula (= Verticicladium trifidum Preuss) is a common colonizer of pine needles (Martinović et al. 2016) but also found as an endophyte in stems and xylem of pine (Petriini and Fisher 1988). The anamorphs fructify after needles fall through needle stromata (Maanen and Gourbière 1997) and apothecia occur on decaying needles. The increased occurrence of *D. acicula* in apparently healthy and symptomless trees in comparison with diseased pines could be explained by competition with other fungal strains colonizing the diseased twigs. The latter may inhibit the growth of *D. acicula* from its common niche, pine needles, into the shoots.

With regard to forest protection, the frequency of Xylariales species (9%, 19 species) is important, especially of Biscogniauxia mediterranea (frequency 2%, found in 49% of the studied sites) and Biscogniauxia nummularia (frequency 0.7%, found in 39% of the studied sites). Both species are known endophytes (Nugent et al. 2005), colonizers of hardwood, and pathogens that take advantage of the weakness of their hosts. They prefer warmer temperatures and seem to benefit from climate warming. In this study, *B. mediterranea* had a higher frequency in pine stands with oak trees or with neighboring oaks in the warmer climate of south Germany than in northern parts. Because it also causes Charcoal canker on Quercus suber L. and other hardwoods in countries of the Mediterranean Basin (Ragazzi et al. 2011; Henriques et al. 2014a, 2014b, 2015), the occurrence of *B. mediterranea* as an endophyte in pine could, in view of climate warming, be evaluated as a potential risk for neighboring oaks in the same forest stand. *B. nummularia* is known to fructify (anamorphic and teleomorphic) only on beech species (Fagus sylvatica and F. orientalis Lipsky) in Eurasia (Læssoe et al. 1999). It is a common endophyte of European beech, although it can induce severe damage, e.g., strip-cankers and wood decay on trees stressed by drought (Greenhalgh and Chesters 1968; Luchi et al. 2015). The occurrence as an endophyte is not confined to beech, as it was also found symptomless in pine, fir, and Douglas fir (authors own, unpublished results) and in other tree species (Petriini 1985). In Northwestern Germany, beech is very often planted as advance regeneration under pine or is associated with pine. In view of global warming, *this* leads us to assign *B. nummularia* in pine as a potential risk for neighboring beech.

The diversity and number of fungal species detected from pine twigs in this study is higher compared with other studies on endophytes in twigs of *P. sylvestris*, where between 10 and 44 species were recorded (Petriini and Fisher 1988; Kowalski and Kehr 1992; Peröhn et al. 2010; Martínez-Álvarez et al. 2012; Sanz-Ros et al. 2015). One explanation for this difference seems to be the much higher number of twigs sampled in this study. Whether the isolation of endophytes on artificial culture medium fully reflects the natural colonization of pine twigs remains questionable. The reason for the low isolation rate of fungi in the Basidiomycota could not be clearly determined in the present study. Plausible explanations for these results in our endophyte isolation study may be that (1) few Basidiomycota fungi live in this ecological niche, (2) sampling and media culturing methods for isolation favored fungi in the Ascomycota, or (3) surface disinfestation of twigs was not effective in killing hyphae of fast-growing epiphytic fungi. Point three was evaluated in a study by Bußkamp (2018) and could be refuted.

In other comparable culture-based studies on endophytes in branches of Scots pine, no fungi in the Basidiomycota were detected (Petriini and Fisher 1988; Kowalski and Kehr 1992; Persöhn et al. 2010; Martínez-Álvarez et al. 2012; and Sanz-Ros et al. 2015). In an endophyte study of Scots pine and mistletoe by Persöhn et al. (2010), which used a culture-based isolation method, only a single basidiomycetous fungus was detected in the mistletoe. In a second study by Persöhn (2013), who worked with direct sequencing of plant tissue, the detection and identification of fungi in the Ascomycota and Basidiomycota were similar.

There is evidence that some endophytes cannot be cultured on nutrient medium (Allen et al. 2003; Arnold 2007; and Unterseher et al. 2007). Numerous studies have shown that Basidiomycetes, in particular, are undetectable on nutrient medium (Kowalski and Kehr 1992; Hoff et al. 2004; Lygis et al. 2005; Menkis et al. 2006; Zamora et al. 2008; Botella and Diez 2011; Sanz-Ros et al. 2015), which is why the detection of endophytes with the help of direct sequencing could be helpful. Ascomycetes that are not cultivatable could thus also be detected (Arnold et al. 2007; Rajala et al. 2013, and Sanz-Ros et al. 2015). Extraction of total DNA of studied tissues could be examined to better determine endophyte communities (Rajala et al. 2013). But the fact that this method cannot distinguish between endo- and epiphytes can be problematic, since surface sterilization may not destroy the DNA of the epiphytes (Schulz and Boyle 2005). A direct comparison between culture-based and molecular methods using needles from *P. taeda* was carried out by Arnold et al. (2007). Their samples were cultured on malt extract agar medium and analyzed with ITS and LSU rDNA. Their results suggest that when isolated on nutrient medium, fungi in the
Basidiomycota were underrepresented, compared with results obtained by direct sequencing methods (Arnold et al. 2007). In contrast, Sordariomycetes were not adequately detected by direct sequencing of plant tissue (Arnold et al. 2007). Fungi in the taxonomic class Sordariomycetes are common endophytic fungi in plant tissue and comprised 31% of all isolations in the present study. Similar results were shown in studies by Sanz-Ros et al. (2015), in which Sordariomycetes accounted for 32% of all isolations from pine branches.

Gazis et al. (2011) argue that many endophyte studies deal only with a single DNA locus. The fungal barcode ITS region is generally used to identify species presence, as in the present work. Advantages of focusing on the ITS region are that this marker is simple to amplify and that, due to its frequent use, many sequences for comparison are available in public data repositories. However, the use of ITS sequences poses problems, as it sometimes shows high intraspecific variation (Lacap et al. 2003). The use of the ITS sequence as barcode region of the fungal genome is insufficient in very diverse genera or species complexes (Lacap et al. 2003; Hoffman and Arnold 2008). The limits of using the ITS region for species delimitation were apparent in the present study, e.g., for the species of the genus Pestalotiopsis Steyaert (Maharachchikumbura et al. 2011, 2014) or the genus Diaporthe (Gomes et al. 2013). Another problem resulting from the use of ITS sequences poses these problems, as it sometimes shows high intraspecific variation (Lacap et al. 2003). The use of the ITS sequence as barcode region of the fungal genome is insufficient in very diverse genera or species complexes (Lacap et al. 2003; Hoffman and Arnold 2008). The limits of using the ITS region for species delimitation were apparent in the present study, e.g., for the species of the genus Pestalotiopsis Steyaert (Maharachchikumbura et al. 2011, 2014) or the genus Diaporthe (Gomes et al. 2013). Another problem resulting from the use of ITS sequences poses these problems, as it sometimes shows high intraspecific variation (Lacap et al. 2003). The use of the ITS sequence as barcode region of the fungal genome is insufficient in very diverse genera or species complexes (Lacap et al. 2003; Hoffman and Arnold 2008).

In summary, a foundational baseline to describe naturally occurring fungal endophyte communities of Scots pine twigs in Germany was established. In the future, it may be possible to identify potential endophytic fungi that suppress S. sapinea.

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Dedication The results are published in honor of Prof. Dr. Franz Oberwinkler, † 15 March 2018, Tübingen in memory of his 80th birthday, 22.05.2019.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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