Ash dieback

The pathogen *Hymenoscyphus fraxineus* and other fungi associated with stem collar necroses of common ash

Eschentriebsterben

Der Erreger *Hymenoscyphus fraxineus* und andere mit Stammfußnekrosen der Gemeinen Esche assoziierte Pilze

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Contents

Abstract

The ascomycete *Hymenoscyphus fraxineus* has caused devastating damage to the common ash (*Fraxinus excelsior*) in Europe in recent decades, resulting in considerable economic and ecological losses. The ash dieback pathogen is native to Asia, where it lives mainly as a saprobiont in peaceful coexistence with the Asian ash species *Fraxinus mandshurica*. Symptoms of the fungus in common ash include wilting of the leaves with subsequent dieback of shoots, twigs and branches, as well as damage to the stem base. These stem collar necroses contribute significantly to the mortality of ash trees. After the mostly primary infection by *H. fraxineus*, secondary wood-destroying fungi can colonise the stem base and the roots. Reduced stability leads to hazards for forest workers and forest visitors, as well as massive dieback and fallen ash trees. Stem collar necrosis is therefore an important aspect of ash dieback and requires comprehensive research.

In the studies of this work stem collar necroses of ash trees were examined in the mycological laboratory. A culture-based method was used to determine and identify the isolated fungi morphologically and by DNA. The results showed, that the composition of the fungal community was different in each stem collar necrosis and at each sampling site due to the large number of species by single isolates. However, fungi of the Ascomycete division made up the largest proportion. The fungi associated with the examined stem collar necroses also included fungal species not previously described on common ash, such as *Cryptostroma corticale*, the causal agent of maple sooty bark disease, or the recently described species *Vexillomyces fraxinicola*, which was found for the first time. Nevertheless, the most common isolated species were also frequently recognised in other studies and were present at most of the sampling sites investigated in Germany. In addition to the ash dieback pathogen *H. fraxineus*, these include especially *Armillaria* species and *Diplodia fraxini*. As weak parasites or pathogens, they can lead to an extension of the damage – *Armillaria* spp. in particular due to its ability to cause intense white rot and *D. fraxini* because of its nature as a very aggressive pathogen.

Multilocus genotype analyses showed, that multiple infections of the stem collar by different strains of *H. fraxineus* are not necessarily related to the size of the stem collar necrosis or the progression of damage. However, they are an important indication for the infection pattern of the pathogenic fungus. The multiple dominance of one genotype per stem collar necrosis suggests that a single strain of *H. fraxineus* initiates the stem collar necrosis and that other strains become established over time through successful infections. The role of

the number of genotypes, the pathogenic aggressiveness of the individual strains or even intraspecific competition should be investigated in further studies.

Zusammenfassung

Der Ascomycet *Hymenoscyphus fraxineus* hat in den letzten Jahrzehnten in Europa verheerende Schäden an der Gemeinen Esche (*Fraxinus excelsior*) verursacht, die zu erheblichen wirtschaftlichen und ökologischen Verlusten geführt haben. Der Erreger des Eschentriebsterbens stammt ursprünglich aus Asien und lebt dort hauptsächlich als Saprobiont in friedlicher Koexistenz mit der asiatischen Eschenart *Fraxinus mandshurica*. Zu den Symptomen, die er bei der hier heimischen Esche hervorruft, gehören neben der Blattwelke mit anschließendem Absterben von Trieben, Zweigen und Ästen auch Schäden am Stammfuß. Diese sogenannten Stammfußnekrosen tragen wesentlich zur Mortalität der Eschen bei. Nach der meist primären Infektion durch *H. fraxineus* besiedeln sekundäre, zum Teil auch holzabbauende Pilze den Stammfuß und die Wurzeln. Die dadurch verminderte Standfestigkeit führt zu einer Gefährdung von Waldarbeitenden und Waldbesuchenden sowie zu massivem Absterben und Umstürzen von Eschen. Die Stammfußnekrosen sind damit ein wichtiges Merkmal des Eschentriebsterbens und bedürfen einer umfassenden Erforschung.

In den Untersuchungen dieser Arbeit wurden Stammfußnekrosen von Eschen im mykologischen Labor untersucht. Die isolierten Pilze wurden mit einer kulturbasierten Methode morphologisch und anhand von DNA bestimmt. Die Ergebnisse zeigten, dass die Zusammensetzung der Pilzgemeinschaft in jeder Stammfußnekrose und an jedem Standort aufgrund vieler Einzelisolate unterschiedlich war. Den größten Anteil machten jedoch Pilze der Abteilung Ascomycota aus. Unter den mit Stammfußnekrosen assoziierten, untersuchten Pilzen befanden sich auch Pilzarten, die bisher noch nicht an Gemeiner Esche beschrieben wurden, wie zum Beispiel der Erreger der Rußrindenkrankheit des Ahorns *Cryptostroma corticale* oder die erstmalig entdeckte und neu beschriebene Pilzart *Vexillomyces fraxinicola*. Die am häufigsten isolierten Arten wurden jedoch auch in anderen Studien als häufig beschrieben und waren auch an den meisten der untersuchten Standorte in Deutschland mit Esche assoziiert. Neben dem Eschentriebsterben-Erreger *H. fraxineus* gehören vor allem *Armillaria* Arten und *Diplodia fraxini* dazu. Beide können als Schwächeparasiten bzw. als Pathogene zu einer Ausweitung der Schäden führen –

Hallimasch insbesondere durch seine Fähigkeit, eine intensive Weißfäule hervorzurufen und *D. fraxini* durch seine Eigenschaften als höchst aggressives Pathogen.

Multilocus Genotyp Analysen zeigten, dass Mehrfachinfektionen des Stammfußes durch verschiedene *H. fraxineus* Stämme nicht unbedingt in einem Zusammenhang mit der Größe oder dem Schadensfortschritt einer Stammfußnekrose stehen. Sie sind jedoch ein wichtiger Hinweis auf das Infektionsmuster des Erregerpilzes. Die häufige Dominanz eines Genotyps pro Stammfußnekrose legt die Vermutung nahe, dass ein einzelner *H. fraxineus* Stamm die Stammfußnekrose auslöst und sich im Laufe der Zeit sukzessive weitere Stämme durch erfolgreiche Infektionen etablieren. Ob und welche Rolle dabei die Anzahl der Genotypen, die pathogene Aggressivität der einzelnen Stämme oder auch die intraspezifische Konkurrenz spielt, sollte in weiteren Studien untersucht werden.

Aim of the work

Stem collar necroses are common in trees affected by ash dieback. They play an important role in the mortality of ash trees. At the start of the project FraxCollar (subproject of the FraxForFuture demonstration project) in the year 2020, the cause and development of stem collar necroses were relatively unknown. It has been suggested that they may also be caused by the ash dieback pathogen *H. fraxineus*. The aim of the subproject and this work was therefore to investigate the aetiology of stem collar necroses on ash and to gain insight into the composition and interaction of the associated fungi and the potential correlation of stem collar necroses with various environmental factors, such as site conditions. These results are important for future silvicultural management of ash or for the development of biological control measures. Other areas of research related to ash dieback (economics, monitoring, genetics, silviculture, viruses and other parts of pathology) were covered in further sub-projects by project partners throughout Germany. The close networking between the various sub-projects also led to collaboration with the FraxGen sub-network (genetics), resulting in the second manuscript “The fungal predominance in stem collar necroses of *Fraxinus excelsior* – a study on *Hymenoscyphus fraxineus* multilocus genotypes”.

The first step in the research process of FraxCollar was the mapping of numerous stem collar necroses across Germany and the assessment of crown damage due to ash dieback on these trees according to Peters et al. (2021a). In a second step, the entire stem base of representative ash trees from different stands was removed and examined in the mycological laboratory of the Northwest German Forest Research Institute. The fungi were isolated mainly from the edges of stem collar necroses at the transition from live to dead wood. A culture-based method was used to determine and identify the isolated fungi morphologically and by DNA. They were characterised as far as possible in terms of their ecological function (pathogens, endophytes, weak parasites, secondary pathogens, wood-decay fungi). In addition to the influence of site factors, emphasis was placed on analysing the role of *H. fraxineus* in the cause and development of necroses. The results of the work lead to a better understanding of the cause and development of stem collar necroses and also provide general information on the fungal biodiversity in the woody tissues of ash trees. These new findings represent an important milestone in the study of ash dieback, stem base necroses and common ash.

1. General introduction

1.1. Common ash (*Fraxinus excelsior*)

The genus *Fraxinus* belongs to the olive family (*Oleaceae*) and comprises 65 species native to the temperate and subtropical latitudes of the Northern Hemisphere (Roloff und Bärtels 2008). Three of these ash species can be found in Germany: Common ash or European ash (*Fraxinus excelsior* L.), manna ash (*Fraxinus ornus* L.), and narrow-leaved ash (*Fraxinus angustifolia* Vahl) (FRAXIGEN 2005). The only species native to Germany is *F. excelsior*, while *F. angustifolia* is native to southern Europe, northern Africa, and western Asia (Meusel et al. 1978). Manna Ash is native to the eastern Mediterranean region and is considered a naturalised neophyte in Germany (Zimmermann et al. 2014; USDA, Agricultural Research Service 2024). Common ash is the most widespread of the three species, occurring throughout northern Europe, south to Turkey, Syria and northern Iran, and east to continental Russia (FRAXIGEN 2005; Roloff and Bärtels 2008). It is one of the few commercial tree species in Germany and is therefore a valuable native deciduous tree species used in forestry, along with European beech and oak. It is one of the tallest deciduous trees in Europe and can grow to a height of 40 metres. Under favourable conditions, ash can live for up to 300 years (Godet 2007). It is often found in fresh mixed broadleaved or riparian forests, along mountain streams and on seeped slopes (Spohn et al. 2005; Kutschera und Lichtenegger 2013). Common ash is also widespread on base-rich sites and often the dominant species on calcareous soils (Ellenberg and Leuschner 2010; Thomas 2016). It clearly has a large site amplitude and can adapt well to different site conditions (Roloff 2013). This robust tree species was also seen as a good candidate for the future in terms of climate change, due to its high level of drought tolerance (Schmidt 2007).

Ash is a dioecious species and its flowers can be both hermaphrodite and unisexual, male or female, on the same tree, but there are also purely male and female trees (Wallander 2001). On average, ash trees produce fruit about once every two years. However, the ash wingnuts, which are abundant at this time, are only released by strong winds in winter. They are dispersed up to a distance of about 150 metres and can lead to dense natural regeneration (Spohn et al. 2005; Roloff 2013). The economic importance of *F. excelsior* is mainly due to its special wood properties and good wood quality. Its wood is ring porous and has high tensile and bending strength. The strong, flexible and durable wood is used for tool handles, sports equipment, furniture, and flooring (FRAXIGEN 2005; Godet 2006; Godet 2007). It is a

heartwood tree with an irregularly coloured core. The absence of ash would not only mean the loss of a wood with these special properties, but also the loss of one of the few economically valuable tree species in Germany, with a corresponding impact on the economy and biodiversity.

1.2. Ash dieback and the causal agent *Hymenoscyphus fraxineus*

Ash dieback was first observed in Poland and Lithuania in the 1990s (Przybył 2002; Lygis et al. 2005). According to Heydeck et al. (2005), first symptoms have been observed in Germany since 2002. In 2006, the pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, & Hosoya was identified by Schumacher et al. (2007). However, initially only the anamorphic stage *Chalara fraxinea* T. Kowalski was found (Kowalski 2006) and mistakenly assigned to the teleomorph *Hymenoscyphus albidus* (Gillet) W. Phillips (Kowalski and Holdenrieder 2009). These two closely related *Hymenoscyphus* species are morphologically almost identical, so the "new" pathogenic species was initially described as the "Falsches Weißes Stängelbecherchen" *Hymenoscyphus pseudoalbidus* V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber & O. Holdenrieder (Queloz et al. 2011). Finally, it was proven that the ash dieback pathogen is the already known Asian species *H. fraxineus* (Baral et al. 2014).

Hymenoscyphus fraxineus is taxonomically placed in *Pezizellaceae, Helotiales, Leotiomycetidae, Leotiomycetes, Pezizomycotina, Ascomycota, Fungi*. The natural range of this non-native, invasive fungus is East Asia. There it is not pathogenic to its natural host tree, *Fraxinus mandshurica* Rupr. and causes little or no damage (Zhao et al. 2012; Zheng and Zhuang 2013). Instead, the fungus is mainly a saprophyte of ash leaves in its natural habitat (Cleary et al. 2016). Thus, the ecological niche of *H. fraxineus* in Asia is similar to that one of *H. albidus* in Germany. Phylogenetic studies suggest that *H. albidus* is a sister species of the Asian *H. fraxineus*, and that *H. albidus* arrived in Europe much earlier (Baral and Bemmann 2014). While *H. fraxineus* is classified as pathogenic and virulent in Europe, its sister species *H. albidus* never occurs as a pathogen (Queloz et al. 2011). Although *H. albidus* has been known in Europe for a long time as a decomposer of ash litter since the beginning of ash dieback this fungal species has been found only rarely or not at all. This suggests that *H. fraxineus* is not only causing severe damage to native ash trees, but is also displacing the native fungal species *H. albidus* (Baral and Bemmann 2014; Kosawang et al. 2023).

The invasive ascomycete *H. fraxineus* has spread very rapidly and successfully over large parts of Europe. The introduction of *H. fraxineus* into Europe is not well understood, but infected nursery stock is thought to have been an important factor in its introduction and spread (Kirisits et al. 2010; NW-FVA 2016; Cleary et al. 2016). According to Agan et al. (2023), the fungus had been introduced into northern Europe years before the first observations of ash dieback in Poland. Gross et al. (2014) observed that the diversity of *H. fraxineus* is significantly higher in Asian populations than in Europe, suggesting that the fungus went through a genetic bottleneck when it became established in Europe. Therefore, only a few individuals of the pathogen appear to have been sufficient to cause the ash dieback epidemic (Gross et al. 2014; Queloz et al. 2017).

Ash dieback and its aftereffects are responsible for the death and drastic decline of ash populations in European forests. The pathogen affects ash trees of all ages in all locations. (Schumacher 2011). *Fraxinus excelsior* is highly susceptible to the pathogen *H. fraxineus*, making ash dieback the most serious threat to common ash so far. *Fraxinus angustifolia* appears to be less susceptible and *F. ornus* does not appear to be affected by the disease (Kirisits et al. 2009).

Hymenoscyphus fraxineus produces its fruiting bodies in early summer on pseudosclerotised rachis from previous years in the leaf litter. The infected rachis are an important reservoir and inoculum in which the fungus can survive for several years (Gross and Holdenrieder 2013). Very small, white, cup-shaped (apothecium) and stalked fruiting bodies (Fig. 1) produce numerous airborne ascospores. A major contributor to the rapid spread of the disease was the dispersal of ascospores through the air (Timmermann et al. 2011). The primary point of entry for the ascospores of the pathogen are the leaves of ash trees. After successful infection, the fungal mycelium can grow into the shoots and spread further into the host tissue (Kräutler and Kirisits 2012; Gross et al. 2012). The characteristic and eponymous symptom of ash dieback can then be seen as infected branches die (Fig. 2). Annual infections lead to a progressive dieback of the crown from the outside to the inside. Besides symptoms such as wilting leaves, dead shoot tips, bark necrosis and discolouration, infections at the base of the stem, called stem collar necrosis, can also occur (Husson et al. 2012; Kräutler and Kirisits 2012; Langer 2017). Secondary damage in the context of ash dieback is mainly caused by insects such as ash bark beetles (*Hylesinus* spp.) or secondary fungi such as various *Armillaria* species (Lenz et al. 2016).

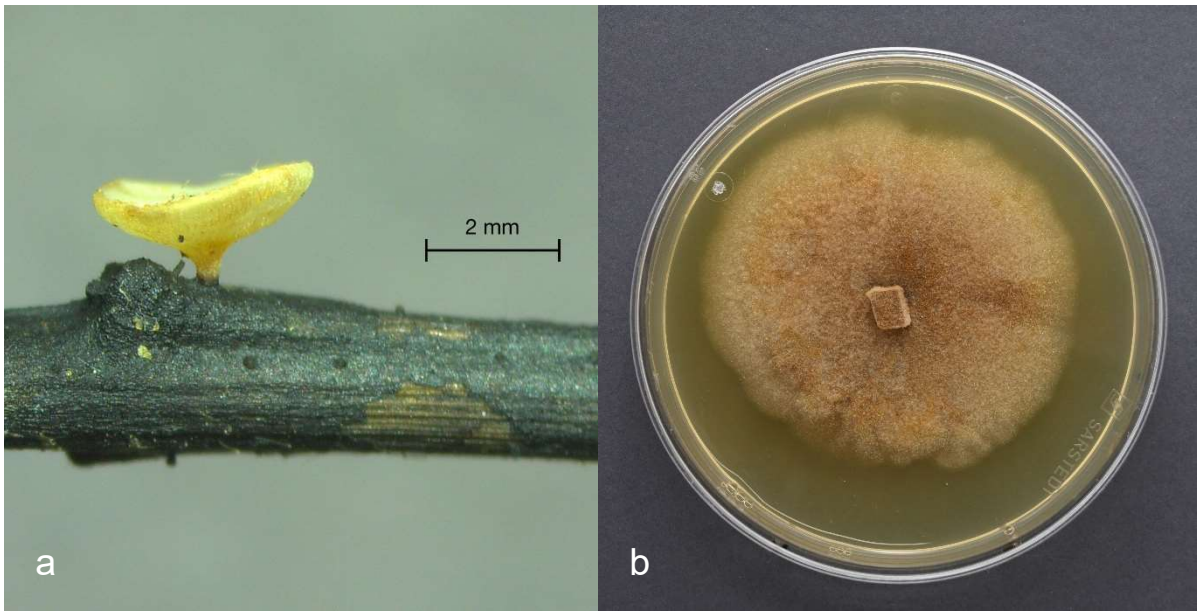

Figure 1: Fruiting body (a) and culture (b) of Hymenoscyphus fraxineus (a) The characteristics of the fruiting bodies are that they are stalked, cup-shaped (apothecium), very small, and white in colour (b) The mycelium of the causal agent of ash dieback is very variable in culture. It can range from light beige to dark brown. However, it can be easily identified by its golden glow on MYP agar. Photo: Sandra Peters.

Since the beginning of ash dieback, the widespread decline of ash trees has threatened its position as a commercial tree species in Germany and has also had a serious impact on the biodiversity of ash-dominated forest ecosystems (Pautasso et al. 2013). In addition, there are high financial losses for the forestry and timber industry. The progressive dieback of infected ash trees is associated with a decline in wood quality and a significant increase in tree mortality (Peters et al. 2021b). The effort required for road and occupational safety increases, as does the risk of accidents to forest workers and visitors due to the lack of stability and resistance to breakage (Langer et al. 2015a). Only a small proportion of the ash population, estimated to be between 1% and 5%, appears to be tolerant to the ash dieback pathogen (McKinney et al. 2011; McKinney et al. 2014). At this time it is not recommended to plant common ash until genetically resistant planting material becomes available. Therefore, the future of ash in European forestry is currently uncertain. However, according to Goberville et al. (2016), there is hope that climate change may mitigate the ash dieback pandemic by decoupling the spatial distribution of the pathogen from that of the common ash.

Figure 2: A crown of an ash tree severely affected by ash dieback. Characteristic symptoms such as dead shoot tips, epicormic shoots, and structural defects can be seen. Following the guidelines of the ash dieback classification key of Peters et al. (2021a), this crown is assessed as damage class 3 (severely damaged). Photo: Sandra Peters.

1.3. Stem collar necrosis as ash dieback symptom

The symptoms of ash dieback are not limited to crown damage. Besides dead shoots, lesions at the base of the stem (stem collar necrosis, Fig. 3) are also a typical symptom of infected ash trees. According to Burokiene et al. (2015), no genetic differences were found between *H. fraxineus* isolates from stem collar necroses and isolates from rachis. This suggests that, in addition to infection of ash leaves, all genotypes of *H. fraxineus* have the potential to cause stem collar necroses (Burokiene et al. 2015). The ability of *H. fraxineus* ascospores to invade

the stem base or roots was also confirmed by Meyn et al. (2019), and the fungus has also been isolated from stem collar necroses in numerous other studies. It is therefore clearly involved as a primary pathogen in the cause and development of stem collar necroses (Husson et al. 2012; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). However, according to Langer et al. (2015b), there are two other types of stem collar necroses in addition to those caused primarily by *H. fraxineus*: Stem collar necroses caused primarily by *Phytophthora* spp. and by soil-borne wood decay fungi such as *Armillaria* spp. The latter also played an important role in

Figure 3: Typical stem collar necrosis caused by the pathogen Hymenoscyphus fraxineus on this common ash. The sunken, discoloured and cracked bark is characteristic for this damage pattern. At this advanced stage, secondary fungi are usually also involved. Due to its reduced stability, this tree is expected to fall in the near future, like the ash tree in the background on the right. Photo: Sandra Peters.

the stem collar necroses analysed in the studies carried out in this work, but *Phytophthora* species were not detected.

Stem collar necroses are most commonly found on ash trees with severe crown damage, but they can also occur on apparently healthy ash trees (Enderle et al. 2017). These observations were confirmed in studies of the FraxCollar subproject of the FraxForFuture demonstration project (Langer et al. 2022). The modular assessment of the ash dieback classification key developed in the project according to Peters et al. (2021a) provides a classification of stem collar necroses into no (stage 0), weak (stage 1) and severe (stage 2) stem collar necrosis. In particular, severe stem collar necrosis (stage 2) is one of the most important mortality factor in ash dieback due to its advanced level of damage (Fuchs et al. 2024). Site conditions, such as soil water supply, also have a certain influence on the occurrence and severity of stem collar necroses. Initial assumptions at the beginning of ash dieback were that wetter forest sites are more likely to have higher frequency and severity of stem collar necroses than drier sites. This has now been confirmed by a number of different studies (Marcais et al. 2016; Chumanová et al. 2019; George et al. 2022; Fuchs et al. 2024).

The role of *Armillaria* spp. and *Diplodia fraxini* in stem collar necroses

In addition to the site conditions, secondary fungal pathogens play an important role in the development of stem collar necroses on ash. The decomposition of roots and the stem base is massively accelerated by secondary colonisation with wood decaying fungi (Heinzelmann et al. 2023). *Hymenoscyphus fraxineus* was more frequently isolated from younger than from advanced stem collar necroses (Enderle et al. 2017). This suggests that the slow-growing primary coloniser is overgrown by secondary fungi in later stages (Matsiakh et al. 2017). Secondary fungi in advanced necroses often include species of the genus *Armillaria*. They occupy a special position among the secondary pathogens associated with ash dieback. In addition to actual ash dieback, *Armillaria* infestation is another serious threat to common ash, as it can contribute significantly to the dieback and thus further reduce the survival of ash trees. The root decay caused by white rot of *Armillaria* spp. can cause affected ash trees to lose stability and fall even in good weather without wind (Heinzelmann et al. 2023). *Armillaria* species are distributed worldwide and occur both as a weak parasite and as a pathogen (Kubiak et al. 2017). The soilborne basidiomycete is very abundant in the soil and this is conducive to infection (Lenz et al. 2016). On already weakened ash trees, species of the genus *Armillaria* can even cause primary stem collar necroses (Langer 2017). Nevertheless, *Armillaria* species, which are known as weakness parasites, dominate in the

context of ash dieback. This suggests that *Armillaria* spp. only attack ash trees that have already been weakened by dieback in the crown or by stem collar necrosis caused by *H. fraxineus* (Heinzelmann et al. 2023).

Another fungal species that has been isolated frequently isolated from stem collar necroses, often even as the dominant species, is *Diplodia fraxini* (Fr.) Fr. (Linaldeddu et al. 2022; Peters et al. 2023). This fungus first came to focus as a frequent coloniser of *F. excelsior* in the context of ash dieback and was initially often misidentified as *Diplodia mutila* (Fr.) Mont. (Kowalski et al. 2016; Linaldeddu et al. 2020). It is possible that infection with *D. fraxini* is facilitated by previous colonisation with *H. fraxineus*. Regardless of the cause of the increased occurrence of *D. fraxini*, this pathogen plays an important role in the damage caused by the ash dieback epidemic. Kowalski et al. (2017) classify *D. fraxini* as the second most common pathogenic fungus after *H. fraxineus*. *Diplodia fraxini* is known to be an aggressive pathogen and, like *H. fraxineus*, can cause bark necrosis and wood discolouration. On common ash, the fungus mainly increases the necroses caused by *H. fraxineus* on branches, stems or stem bases (Alves et al. 2014; Linaldeddu et al. 2020; Linaldeddu et al. 2022).

2. Manuscript I

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ORIGINAL ARTICLE

Fungi associated with stem collar necroses of *Fraxinus excelsior* affected by ash dieback

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Abstract

In recent decades the vitality and physical stability of European ash trees in Germany have been reduced by European ash dieback, especially when associated with stem collar necroses and rots. This study was carried out to investigate the composition of the fungal communities associated with stem collar necroses. Filamentous fungi were isolated from 58 ash trees out of nine forest stands in northern, eastern, and central Germany. Obtained isolates were identified to a genus or species level by means of morphological and molecular analyses. In total 162 morphotypes including endophytic, saprotrophic, and pathogenic fungi were isolated. For 33 species found no prior reports from *Fraxinus excelsior* were recognised, including *Cryptostroma corticale* and *Diplodia sapinea*. None of the identified species were found at all studied sites, though *Diplodia fraxini* was the most common fungus with regard to frequency within all isolates, occurring at seven sample sites. This species is followed by *Hymenoscyphus fraxineus*, *Armillaria* spp., *Neonectria punicea*, *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium*, and *Paracucurbitaria* sp. in order of frequency within all isolates. The aforementioned species are characterised and analysed in respect to their occurrence in stem collar necroses and at sample sites. The influence of site conditions on the fungal composition was described for five intensively sampled sites with a minimum of five studied trees (Schwansee, Rhüden, Berggießhübel, Satrup, and Schlangen). The sampling site of Schlangen was further subdivided into four subplots with different positions in the terrain. In the remaining four extensive sample sites, either one or two trees, respectively, were sampled and analysed (Oranienbaumer Heide, Woltershausen, Wolfenbüttel, and Neuhege). Over all sample sites, fungal communities of symptomatic stem tissue are similar concerning the most frequent fungi, but vary greatly according to singularly isolated fungi.

Keywords *Fraxinus excelsior* · Endophytes · Fungal communities · Ash dieback · Stem collar necroses

Introduction

Since the early 1990s, the European ash (*Fraxinus excelsior* L.) is threatened by European ash dieback, caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, & Hosoya (*Helotiaceae*, *Ascomycota*). First disease reports came from Poland and Lithuania (Przybyl 2002; Lygis et al. 2005). In Germany, the severe disease was observed since 2002 and the causal agent was first proven in the year 2006 (Heydeck et al. 2005; Schumacher et al. 2007). Meanwhile, this invasive fungal pathogen has become widespread in Europe. Affected European ash (hereafter referred to as ash) trees of all ages show a broad range of symptoms, such as leaf necrosis, wilting, shoot blight, inner bark discolorations, sunken cankers, epicormic shoots, as well as stem collar, and root necrosis (Gross et al. 2014; Langer 2017). Reduction of

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tree stability and increase of mortality of ash is often connected to stem collar necrosis, which progresses towards xylem and heartwood. Stem collar necrosis can occur on trees with or without crown symptoms of ash dieback, but is often observed on diseased trees (Schumacher et al. 2009; Husson et al. 2012; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). Stem collar necroses are defined as basal lesions with necrotic tissue on the outside and the inside of the stem mainly caused by fungi (Langer 2017). The actual shape of stem collar necroses depends on different factors, such as individual progress or associated fungi. Advanced necroses are often associated with wood rot caused by fungi colonising the stem following the initial infection by *H. fraxineus* (Langer 2017).

Even though many fungi are reported from *F. excelsior* (981 different species according to the USDA website, Farr and Rossman 2022, retrieved on 17.06.2022) fungi associated with necrotic stem tissue of ash, specifically in Germany, have rarely been described (Enderle et al. 2017; Langer 2017; Meyn et al. 2019). Most frequently isolated from the necroses at the collar base of diseased ash trees were *Armillaria* spp., *Diaporthe eres* Nitschke, *Diplodia* spp., *Fusarium avenaceum* (Fr.) Sacc., *F. lateritium* Nees, *Fusarium solani* (Mart.) Sacc. (syn. *Neocosmospora solani* (Mart.) L. Lombard & Crous), *H. fraxineus*, and *Neonectria punicea* (J. C. Schmidt) Castl. & Rossman (Lygis et al. 2005; Langer 2017; Meyn et al. 2019; Linaldeddu et al. 2020). A strong evidence for *H. fraxineus* being the causal agent of ash dieback was given by Chandelier et al. (2016), who proved occurrence of *H. fraxineus* in the majority of symptomatic tissue of ash stem collar. Langer (2017) confirmed frequent isolation from stem collar necroses and the assignment as primary agent. But not every type of stem collar necrosis must be primarily caused by *H. fraxineus*. Langer (2017) showed that basal necroses can be caused by *Phytophthora* under special site conditions, as found in floodplain forests or by *Armillaria* spp. on weakened ash trees. The path of infection by *H. fraxineus* still remains unknown and little is understood about the influence of environmental factors on stem collar necroses. Site characteristics, such as moisture content, are assumed to affect disease severity. Kenigsvalde et al. (2010) and Marçais et al. (2016) determined that disease severity correlates positively with soil humidity conditions. It has been suggested that stem collar necroses development and extent is also related to moist conditions or humid topographical positions (Marçais et al. 2016). Therefore the design of this study covers a wide range of water supply types at sampling sites. The composition of the forest stands combined with their nutrient and water availability could be a factor in assessing differences in fungal diversity per stand. Most trees moderately and severely damaged due to ash dieback were observed at forest sites with a high soil organic matter content and a neutral to slightly alkaline soil pH (Turczański et al. 2019). Hence, it can be suspected that fungal composition depends on soil and water availability just as well (Linaldeddu et al. 2011; Salamon et al. 2020).

This study is conducted as part of the demonstration project FraxForFuture and the sub-network FraxPath (Langer et al. 2022). The aims of this research are to fill knowledge gaps concerning the α-diversity of cultivable *Dikarya* Hibbett, T. Y. James & Vilgalys associated with stem collar necroses of trees affected by ash dieback and the composition of their fungal communities. Therefore, fungi associated with necrotic stem bases of ash were isolated and identified from 58 ash trees in order to determine the continuity and the frequency of *H. fraxineus* and secondary fungi. The role of the most frequent fungi in the process of stem collar necroses formation is discussed.

Materials and methods

Sampling sites

In total, six federal states of Germany (Lower Saxony, North Rhine-Westphalia, Saxony, Saxony-Anhalt, Schleswig-Holstein, and Thuringia) were investigated. Nine mixed broad-leaved forest stands with a substantial share of *F. excelsior* affected by ash dieback were selected in order to cover different sites with a wide range of soil water supply types (Table 1 and Fig. 1). The sample sites are located in northern, eastern, and central Germany with sub-oceanic to sub-continental temperate zones. All sites are eutrophic and cover the most common substrates of ash stands in Germany. Basic soil and geological data were acquired by using geological maps with a high resolution (scale 1:25000) of the respective federal geology departments and the forest inventory and forest site mapping data sets of the federal forestry authorities. Additional data from soil core sampling, soil profiles, and soil analyses were available for the sampling sites of Rhüden, Berggießhübel, Schwansee, and Schlangen because these sites are part of other studies associated with soil inventories: The site of Rhüden is part of the national forest soil inventory. Schwansee and Berggießhübel correspond to the intensive monitoring plots "TH_1 Schwansee" and "SN_2 Bienhof" of the research cluster FraxForFuture (Langer et al. 2022). The largest forest stand continuously including ash trees investigated in this study (Schlangen) has a pronounced relief and was divided into four subplots to investigate different positions in the terrain. In this case, intensive soil exploration was conducted to differentiate between the four subplots, including pedological assessments and soil sampling from soil profiles.

The shallow sites (Rhüden, Schlangen 2) in exposed terrain positions on limestone with high coarse soil fractions and low water storage capacities represent the driest

Table 1 Sampling site information sorted by sampling date; all forest sites are eutrophic

Forest site (coordinates UTM)	Sampling classification (sample number; sample date)	Metres above sea level	Exposition and inclination	Climate	Soil water supply	Soil and bedrock	Mixture of tree species in stand
Oranienbaumer Heide (33 U 318931 5730457)	Extensive sampling (1; 19.10.2020)	90	Flat	Sub-continental	Slightly moist, fluctuating ground water regime	Diluvial sands, boulder clay	*Quercus robur*, *Fagus sylvatica*, *Carpinus betulus*, *Betula pendula*, *Fraxinus excelsior*
Woltershausen (32 U 564973 5757894)	Extensive sampling (2; 20.10.2020 and 04.03.2021)	289	SSE, moderate inclination (9–18°)	Weakly sub-atlantic	Slightly moist	Clayey loam over cretaceous limestone	*Fagus sylvatica*, *Acer pseudoplatanus*, *Fraxinus excelsior*
Wolfenbüttel (32 U 632092 5778288)	Extensive sampling (1; 27.10.2020)	238	SE, slight inclination (0–9°)	Weakly sub-atlantic	Moist, high water holding capacity	Colluvial deposit over upper Muschelkalk (triassic limestone)	*Fagus sylvatica*, *Acer pseudoplatanus*, *Fraxinus excelsior*
Schwansee (32 U 646432 5660792)	Intensive sampling (8; 10.11.2020)	163	Flat	Weakly sub-continental	Gleysol, ground water influenced	Carbonatic lake gravel deposit, lake sediment	*Fraxinus excelsior*, *Quercus robur*, *Populus nigra*, *European alder*
Rhüden (32 U 579914 5757111)	Intensive sampling (10; 22.02.2021)	235	WSW, strong inclination (18–27°)	Weakly sub-atlantic	Slightly dry, well drained, low water holding capacity	Lower Muschelkalk, (triassic limestone) with shallow loess cover	*Fraxinus excelsior*, *Fagus sylvatica*, *Acer pseudoplatanus*, *Prunus avium*
Neuhege (32 U 593054 6010661)	Extensive sampling (1; 08.06.2021)	53	ESE, slight inclination (0–9°)	Moderately sub-atlantic	Stagnosol/gleysol, ground water influenced	Marly till, boulder clay	*Quercus robur*, *Alnus glutinosa*, *Fraxinus excelsior*, *Fagus sylvatica*
Berggießhübel (33 U 426320 5629803)	Intensive sampling (11; 01.07.2021)	475	NNW, slight inclination (0–9°)	Sub-continental, submontane	Slightly moist, slope water influenced, slightly stagnic	Basalt and gneiss solifluction soil with loamy loess cover	*Betula pendula*, *Acer pseudoplatanus*, *Fraxinus excelsior*, *Tilia cordata*, *Quercus robur*, *Prunus avium*, *Sorbus aucuparia*
Satrup (32 U 546373 6072699)	Intensive sampling (5; 06.07.2021)	49	ESE, slight inclination (0–9°)	Moderately sub-atlantic	Slightly moist, high water holding capacity, slightly stagnic	Marly till, boulder clay	*Fagus sylvatica*, *Fraxinus excelsior*
Schlangen 1 (32 U 492453 5740809)	Intensive sampling (3; 01.11.2021)	274	valley bottom, NNW, slight inclination (0–9°)	Moderately sub-atlantic	Moist, high water holding capacity, slightly stagnic	Colluvial deposit, silty loam	*Fagus sylvatica*, *Fraxinus excelsior*, *Acer pseudoplatanus*, *Prunus avium*
Schlangen 2 (32 U 492621 5741084)	Intensive sampling (6; 08.11.2021)	299	upper slope, S, strong inclination (18–27°)	Moderately sub-atlantic	Slightly dry, low water holding capacity	Cretaceous limestone, very shallow loess cover (clayey loam)	*Fagus sylvatica*, *Fraxinus excelsior*, *Acer pseudoplatanus*, *Prunus avium*

Table 1 (continued)

Forest site (coordinates UTM)	Sampling classification (sample number; sample date)	Metres above sea level	Exposition and inclination	Climate	Soil water supply	Soil and bedrock	Mixture of tree species in stand
Schlangen 3 (32 U 492515 5740880)	Intensive sampling (4; 01.11.2021)	305	upper slope, NNW, slight inclination (0–9°)	Moderately subatlantic	Slightly moist, moderate water holding capacity	Cretaceous limestone, moderate loess cover (clayey loam)	*Fraxinus excelsior*, *Fagus sylvatica*, *Acer pseudoplatanus*, *Prunus avium*
Schlangen 4 (32 U 492738 5741014)	intensive sampling (6; 01., 02., 08.11.2021)	316	hill top, W, slight inclination (0–9°)	Moderately subatlantic	Slightly moist, moderate water holding capacity	Cretaceous limestone, moderate loess cover (clayey loam)	*Fagus sylvatica*, *Acer pseudoplatanus*, *Fraxinus excelsior*, *Prunus avium*

end of the ecological niche of ash. Several other sites have moderate (Schlangen 3, Schlangen 4, Wolterhausen) or high water storage capacities (Wolfenbüttel, Schlangen 1) because of medium to deep loamy loess covers or colluvial deposits. The remaining sites are primarily characterised by either stagnic soil conditions (Satrup, Berggießhübel), slope water influence (Berggießhübel), or groundwater influence (Oranienbaumer Heide, Schwansee, Neuhege). Soil substrate, water retention capacity, terrain relief, climate, and presence/absence of groundwater or stagnic soil properties (Table 1) were combined to create a ranking of site water supplies of the sampling sites (Online Resource 1 and Online Resource 2).

Between one and nineteen trees were excavated and sampled per site. Hence, the sampling sites were divided into intensive and extensive sampling sites. The intensive sampling sites had a minimum of five and a maximum of 19 sample trees. Intensive sampling was conducted in Rhüden (10 sampled trees), Satrup (5), Berggießhübel (11), Schwansee (8), and Schlangen (19). Each intensive sampling site or subplot was 0.2–0.5 ha in size. The extensive sampling sites with only one or two examined ash trees were taken into account only for fungal occurrence: Woltershausen (2 sampled trees), Wolfenbüttel (1), Neuhege (1), and Oranienbaumer Heide (1). These individual trees were included to increase the sample set and the distribution of investigated stem collar necroses and their associated fungi.

Sampled trees

In total 58 ash trees were sampled, including six trees initially selected as control trees (two in Berggießhübel, one in Satrup, and three in Schlangen; Online Resource 3). The diameter at breast height of the sampled ash trees ranged from approximately 7–25 cm. The age of the sample trees ranged from 15 up to 80 years. The majority was approximately 40 years old. Classification of stem base and crown condition of the studied trees was carried out according to the guidelines of Peters et al. (2021a, b). Additionally, the neighbouring tree species occurring in the studied stands were noted (Table 1).

Ash trees were felled in the years 2020–2021 and cut at least 15 cm above the visible necrotic area. Subsequently, trunk bases and the uppermost parts of the main roots were dug out with picks and shovels. Depending on soil structure (rock content) final roots were cut by chainsaw with a .325-in. Rapid Duro 3 (RD3), 1.6-mm chainsaw chain (STIHL AG & Co. KG, Dieburg, Germany). The stem collars were transported to laboratory in clean and marked plastic bags.

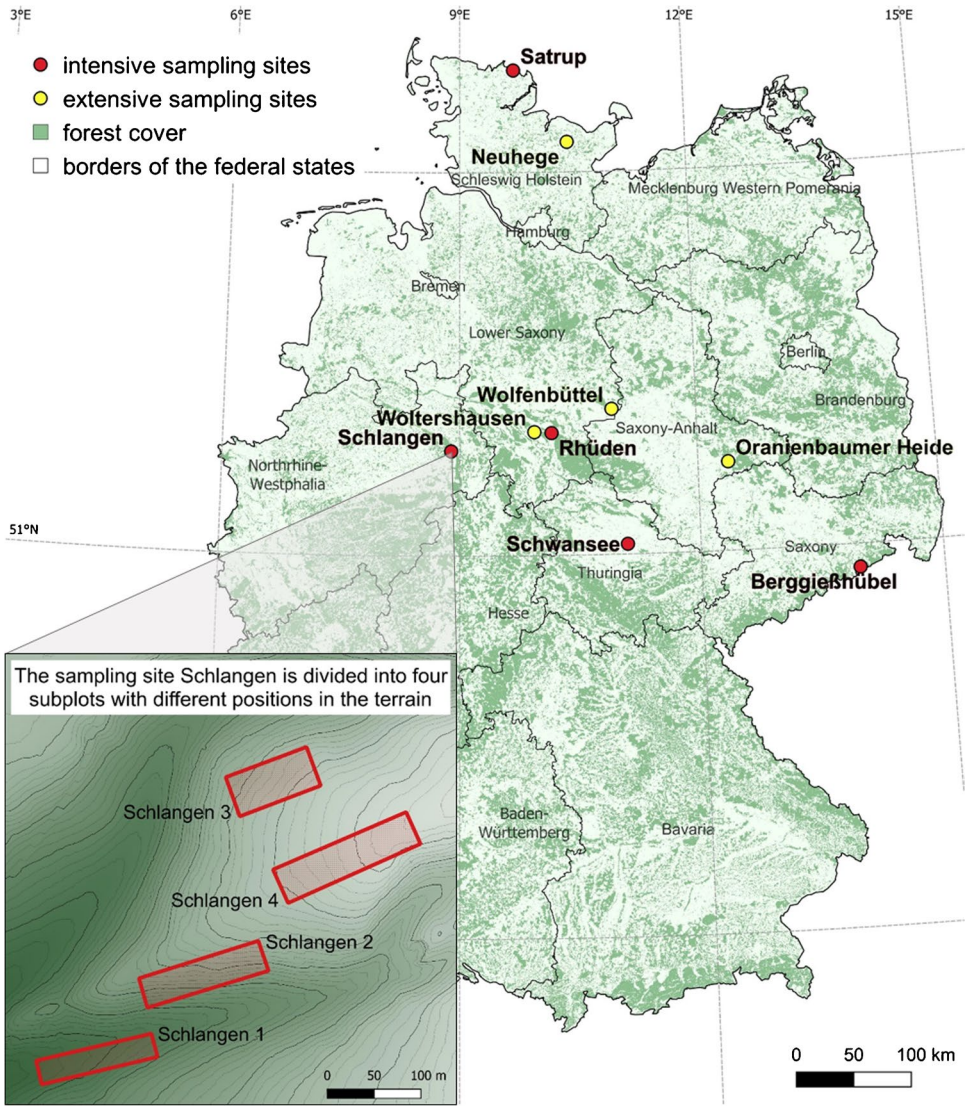

Fig. 1 Sampling sites in Germany divided in intensive (red) and extensive (yellow) sampling sites with a detailed view of the special study site Schlangen and its feature of splitting in four subplots with different terrain positions (resources: QGIS 3.24 © GeoBasis-DE/BKG (2022), DGM1 © GeoBasis NRW (2021))

Isolation of fungi

In preparation for fungal isolation, tree stems were cleaned with a coarse brush under tap water. After air-drying overnight, the samples were processed in the laboratory. Each sample was photographed for documentation. Bark was removed from symptomatic stem areas at the transition zones of living and dead woody tissue. Depending on the thickness of the bark either a sterilised knife or a scalpel was used for removal of the bark. The exposed tissue was sprayed with 70% ethanol all over the necrosis. For chipping of wood tissue samples a chisel and hammer were used. All tools were sterilised by flame shortly before each use. The top tissue layer of the sample was discarded and only the sterile layer directly underneath was incubated. The number of wood chips taken from each tree varied depending on the size of the necrosis. Three of the 5–10-mm-long wood chips were placed in a 90 mm petri dish containing malt yeast peptone (MYP) agar, modified according to Langer (1994) containing 0.7% malt extract (Merck, Darmstadt, Germany), 0.05% yeast extract (Fluka, Seelze, Germany), 0.1% peptone (Merck) and 1.5% agar (Fluka). Once the surface of necroses was processed, stem collars were cut longitudinally with a band saw and carefully sanded for better visualisation of the discolorations. The longitudinal sections were treated according to the isolation method for the surface of necrosis described above. Wood chips were taken at the edge of the necroses and at the transition areas of different discolorations. The process was repeated with the cross section of the stem (Fig. 2).

The petri dishes containing wood chips were incubated at room temperature under ambient daylight for four weeks. The cultures were checked for isolates once a week. Emerging mycelia of filamentous fungi were sub-cultured into pure cultures. The pure cultures were grouped into morphotypes (MT) based on similarity of

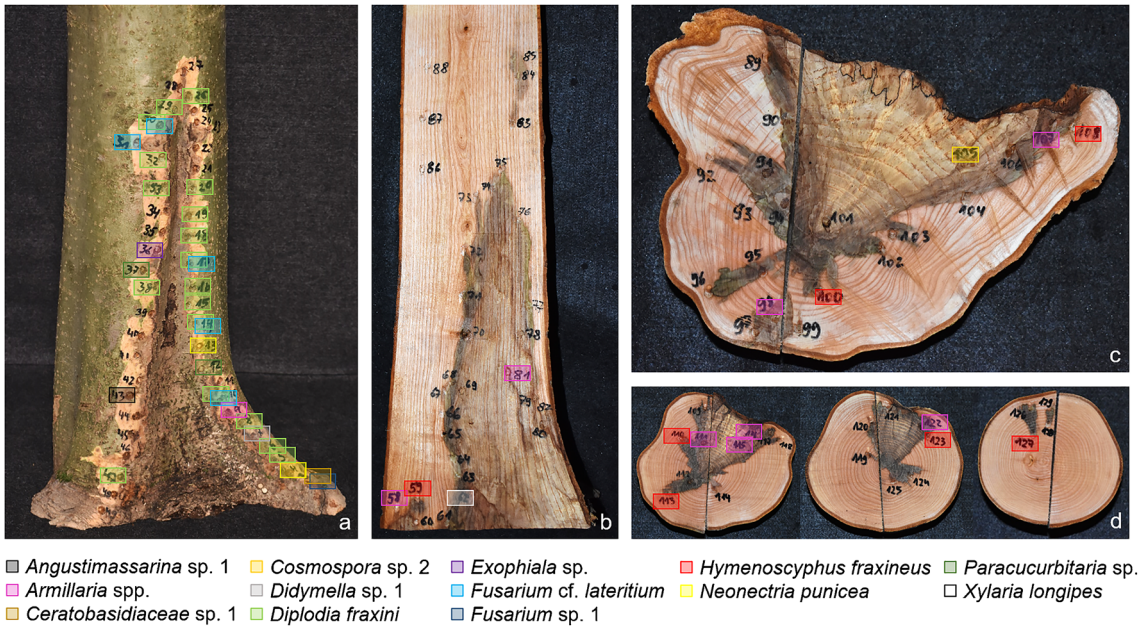

Fig. 2 Fungi associated with the stem collar necrosis of ash tree number 53 (Online resource 3) from the sampling site Schlangen 2. Isolation loci are numbered. **a** Sampled stem base from the outside, **b** longitudinal-section of stem base with visible wood discoloration, **c** basal cross-section of stem base, and **d** cross-section of the stem base above ground level every 10–15 cm

colony morphology. At least one representative culture for each MT was stored in MYP slants at 4 °C at the fungal culture collection of the Northwest German Forest Research Institute (NW-FVA). Beside the MT assignment, contaminated or overgrown fungi were summarised under "Fungus sp.".

The frequency of each isolated fungal MT within all isolates (f_{MT}) was specified as the percentage of this particular MT in all isolates. To measure the ratio of morphotype isolates to isolation attempts, the frequency of isolated fungal MT in relation to the total amount of wood chips (f_{WC}) is used. Continuity of isolated MT is defined as the number of sampled trees where the MT was detected in relation to the total number of sampled trees. Analyses of the fungal diversity found in this study were conducted using RStudio (v. 4.1.2, R Core Team 2021). The packages used were tidyverse (Wickham et al. 2019), ggplot 2 (Wickham 2016), and ggVennDiagram (Gao 2021).

The dependency of f_{WC} of the most commonly isolated fungal MT on site water supply ranks of the intensive sampling sites was tested by using Dirichlet regression (Maier 2014) and the R add-on package DirichletReg (Maier 2021). A non-parametric test is necessary because site water supply is an ordinal variable. The ranks are based on a combination of soil water retention, soil stagnic properties, relief, topography, and climate characteristics of the study sites and correspond to the site descriptions in Table 1 and Online Resource 1.

Molecular analysis

For molecular analysis, at least one representative strain from each MT was chosen. Mycelium was placed in 1.5-ml Eppendorf tubes with three glass beads (3 mm) and 150 µl of TE buffer (10 ml 1 mmol Tris HCl (pH 0.8), 2 ml 0.5 mmol EDTA; Carl Roth, Karlsruhe, Germany), and crushed in a Mixer Mill MM 200 (Retsch, Haan, Germany) with 25 vibrations per second for 90 s. Subsequently, genomic DNA was extracted following the protocol of Izumitsu et al. (2012).

The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers ITS-1 and ITS-2 (ITS region) was amplified for all strains using the primer pair ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990). Additionally, for a selection of strains belonging to *Armillaria*, a partial sequence of the translation elongation factor 1α (*EF-1α*) was amplified using the primer pair EF595F + EF1160R (Kauserud and Schumacher 2001). The PCR mixture consisted of 1 µl of DNA and 19 µl mastermix which contained 2.5 µl 10× PCR reaction buffer (with 20 mM $MgCl_2$, Carl Roth, Karlsruhe, Germany), 1 µl of each primer (10 mmol), 2.5 µl $MgCl_2$ (25 mmol), 0.1 µl Roti®-Pol Taq HY Taq polymerase (Carl Roth, Karlsruhe, Germany) and 2.5 µl of 2 mmol dNTPs (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Each reaction was topped up to a volume of 20 µl by adding sterile water. A StepOnePlus™ PCR System (Applied Biosystems, Waltham,

Massachusetts, US) was used to carry out the DNA amplifications. The PCR conditions for the amplification of the ITS and *EF-1α* regions were set according to Bien et al. (2020) and Guo et al. (2016), respectively. A 1% agarose gel was used to visualise the PCR products. The products were sent to Eurofins Scientific Laboratory (Ebersberg, Germany) for sequencing. Initially, PCR samples of the ITS region were sequenced using the forward reaction (primer ITS-1F). In case of imprecise results, additionally, reverse reactions (primer ITS-4) were sequenced. PCR products of the *EF-1α* sequence region were sequenced by the respective forward and reverse reactions. All resulting sequences were visually checked and edited as follows using BioEdit Sequence Alignment Editor (v. 7.2.5; Hall 1999). Consensus sequences were generated, for all strains with forward and reverse sequences available. Defective sequence beginnings and ends were trimmed and erroneous nucleotide allocations corrected. Sequences were submitted to GenBank (Table 2).

Identification of fungi

Only cultivatable Dikarya fungi were investigated, however, yeasts were not taken into account. Only isolates which are clearly growing from the wood chips were determined. Obvious contamination with filamentous fungi was not considered. The genus *Trichoderma* was not included in the analyses, because it is difficult to assess whether these very fast-growing fungi were contaminations or real outgrowth from the wood.

MT were assigned to fungal taxa based on morphological observation and molecular analysis of representative strains following the method of Guo et al. (2000). For fungal taxon determination blastn searches based on ITS sequences were conducted on the GenBank database (http://www.ncbi.nlm.nih.gov/genbank, Altschul et al. 1997) excluding uncultured/environmental sample sequences from the search. Results were critically interpreted with emphasis on well-curated culture collections, such as the Westerdijk Fungal Biodiversity Collection (CBS). In general, blastn results on a species level below a threshold of 98% identity were not trusted to be accurate enough for final determination. In case no definite affiliation to a specific taxonomic level was possible, for example because of more than one hit with a threshold of over 98% identity, the identification was marked by cf. (confer) to indicate uncertainties. The results were rechecked against literature and previously identified cultures from the institute's collection for confirmation. In addition to blastn searches, extended analyses for taxon determination on a species level were conducted for isolates belonging to *Diplodia* and *Armillaria* due to the considerable number of isolates from these genera. Phylogenetic analyses were conducted based on an ITS sequence-dataset and an ITS-*EF-1α* concatenated sequence-dataset for *Diplodia* and *Armillaria* isolates, respectively, including appropriate reference sequences retrieved from GenBank. Both analyses were performed using RAxML v. 8.2.11 (Stamatakis 2006, 2014) as implemented in Geneious R11 (Kearse et al. 2012) using the GTRGAMMA model with the rapid bootstrapping and search for best scoring ML tree algorithm including 1000 bootstrap replicates (Online Resource 4 and 5).

As a rule, current names were applied according to the nomenclatorial database MycoBank (Robert et al. 2005). Two exceptions from this generally applied rule have been made in the case of the MT designated here as *Fusarium solani* s. l. and *Armillaria gallica*. In the case of *F. solani* the authors are aware that there is a currently unsettled discussion about correct delimitation of this species, or rather species complex. Here we follow the "classic" nomenclature substantiated by Geiser et al. (2021) obverse that promoted by Lombard et al. (2015) and Sandoval-Denis et al. (2019) who place said species complex in the genus *Neocosmospora* (*N. solani* (Mart.) L. Lombard & Crous). The currently applied name for *A. gallica*, according to MycoBank is *A. lutea* Gillet. However, Marxmüller (1992) stated that *A. lutea* is a nomen ambiguum and the later introduced name *A. gallica* Marxm. & Romagn. is to be used (Burdsall and Volk 1993).

Results

Sampled trees

The crown health status regarding ash dieback of the sampled trees ranged from vital and nearly without dieback symptoms to almost dead. All sampled trees (Online Resource 3), except for the six planed control trees, had obvious stem collar necroses through discoloured, sunken, and in some cases ruptured bark. In cases of ruptured bark, subjacent stem collars were neither completely rotting nor dead. During processing of the sample trees in the laboratory two out of the six control trees (trees 32 and 42, Online Resource 3) showed necrotic woody tissue inside the stem base. Consequently, these two trees were transferred to and analysed as sample trees (symptomatic sample trees $n = 54$, asymptomatic control trees $n = 4$).

Isolated fungi

In total, 4401 wood chips of stem collar tissue originating from 58 trees were incubated. A total of 1511 isolates (from which 226 were not identifiable due to contaminations; marked as Fungus sp.) from 1413 wood chips (32%) were observed. 958 chips (22%) showed no outgrowth at all after four weeks of incubation, while 960 (22%) chips had been overgrown by fast-growing fungi from adjacent wood chips

Table 2 List of isolated fungi sorted alphabetically within orders; column "Species" is the name finally determined by the authors; fungi causing wood rot are marked with *; probable first reports of species isolated from *F. excelsior* are marked with [FR]; Phylum: A = *Ascomycota*. B = *Basidiomycota*. Datum Blast: 22./23.06.2022

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
									Basis of identification	ID	Identity (%)	Reference
Acremonium sp.	A	*Hypocreales*	8031	OP023272	1	0.07%	2%	1	*Acremonium variecolor*	KF313108.1	99.63%	Min et al. 2014
Akanthomyces sp.[FR]	A	*Hypocreales*	6958	OP023220	1	0.07%	2%	1	*Akanthomyces muscarius*	MH858126.1	100.00%	Vu et al. 2019
Alternaria infectoria	A	*Pleosporales*	5940	OP023162	3	0.20%	5%	2	*Alternaria infectoria*	MT561399.1	100.00%	Langer and Bußkamp (unpublished)
Alternaria sp.	A	*Pleosporales*	6119	OP023191	5	0.33%	5%	2	*Alternaria angustioovoidea*	MH861939.1	100.00%	Vu et al. 2019
Angustimassarina sp. 1	A	*Pleosporales*	5951	OP023165	2	0.13%	3%	1	*Angustimassarina lonicerae*	KY496759.1	100.00%	Tibpromma et al. 2017
Angustimassarina sp. 2	A	*Pleosporales*	6207	OP023203	4	0.26%	3%	2	*Angustimassarina lonicerae*	KY496759.1	98.61%	Tibpromma et al. 2017
Armillaria spp.*	B	*Agaricales*	5952	OP023166	158	10.46%	50%	5	*Armillaria gallica*	KX618575.1	99.74%	Denman et al. 2017
									Armillaria cepistipes	MK966557.1	99.62%	Wei (unpublished)
Ascocoryne sp. 1*	A	*Helotiales*	7103	OP023244	3	0.20%	3%	2	*Ascocoryne* sp.	MH682237.1	100.00%	Johnston and Park (unpublished)
Ascocoryne sp. 2*	A	*Helotiales*	8030	OP023271	1	0.07%	2%	1	*Ascocoryne solitaria*	HM152545.1	100.00%	Griffin et al. 2010
Aureobasidium pullulans	A	*Dothideales*	6176	OP023196	5	0.33%	7%	3	*Aureobasidium pullulans*	KT693733.1	99.82%	van Nieuwenhuijzen et. al (unpublished)
Beauveria bassiana	A	*Hypocreales*	6979	OP023247	2	0.13%	2%	1	*Beauveria bassiana*	MN122432.1	100.00%	Gasmi et. al (unpublished)
Beauveria pseudobassiana	A	*Hypocreales*	6001	OP023182	1	0.07%	2%	1	*Beauveria pseudobassiana*	NR_111598.1	100.00%	Schoch et al. 2014
*Biscogniauxia nummularia**	A	*Xylariales*	5926	OP023156	3	0.20%	5%	1	*Biscogniauxia nummularia*	NR_153649.1	100.00%	Wendt et al. 2018

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
									Basis of identification	ID	Identity (%)	Reference
Bispora sp.*	A	Incertae sedis	8329	OP023300	1	0.07%	2%	1	*Bispora antennata*	KY462800.1	99.03%	Baral and Marson (unpublished)
*Bjerkandera adusta**	B	*Polyporales*	5943	OP023163	5	0.33%	9%	3	*Bjerkandera adusta*	MK322310.1	100.00%	Chilean Collection of Microbial Genetic Resources (unpublished)
Botrytis cf. *cinerea*	A	*Helotiales*	6874	OP023212	6	0.40%	9%	3	*Botrytis cinerea*	MH860108.1	100.00%	Vu et al. 2019
Cadophora dextrinospora FR	A	*Helotiales*	7026	OP023226	11	0.73%	10%	3	*Cadophora dextrinospora*	NR_119489.1	100.00%	Schoch et al. 2014
Cadophora melinii	A	*Helotiales*	6985	OP023248	1	0.07%	2%	1	*Cadophora melinii*	MH791342.1	99.02%	Alborés 2018
Cadophora ramosa FR	A	*Helotiales*	6194	OP023202	8	0.53%	7%	4	*Cadophora ramosa*	MN232956.1	100.00%	Bien and Damm 2020a
Cadophora sp. 1	A	*Helotiales*	7100	OP023258	3	0.20%	3%	2	*Cadophora malorum*	MT561395.1	100.00%	Langer and Bußkamp (unpublished)
Cadophora sp. 2	A	*Helotiales*	8478	OP023311	1	0.07%	2%	1	*Cadophora malorum*	MT561395.1	100.00%	Langer and Bußkamp (unpublished)
Calycina herbarum FR	A	*Helotiales*	8287	OP023287	1	0.07%	2%	1	*Calycina herbarum*	MZ159660.1	99.42%	Gaya et. al (unpublished)
Camposporium sp.	A	Incertae sedis	8336	OP023307	1	0.07%	2%	1	*Camposporium ramosum*	MH866030.1	96.17%	Vu et al. 2019
Cephalotrichiella penicillata FR	A	*Microascales*	7042	OP023232	1	0.07%	2%	1	*Cephalotrichiella penicillata*	NR_153893.1	99.82%	Crous et al. 2014
Ceratobasidiaceae sp. 1	B	*Cantharellales*	8003	OP023265	2	0.13%	3%	1	*Ceratobasidium* sp.	KX786242.1	99.84%	Lakshman et al. 2017
Ceratobasidiaceae sp. 2	B	*Cantharellales*	8005	OP023267	1	0.07%	2%	1	*Ceratobasidiaceae* sp.	KX610454.1	98.26%	Yokoya et al. 2017
Chaetomiaceae sp.	A	*Sordariales*	8332	OP023303	1	0.07%	2%	1	*Chaetomium* sp.	MK182798.1	99.81%	Li and Xu (unpublished)
Chaetomium globosum	A	*Sordariales*	8326	OP023297	1	0.07%	2%	1	*Chaetomium globosum*	MH858130.1	99.81%	Vu et al. 2019

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
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Chaetomium sp.	A	*Sordariales*	8323	OP023295	1	0.07%	2%	1	*Chaetomium subaffine*	MH862288.1	100.00%	Vu et al. 2019
Chloridium virescens var. *caudigerum*	A	*Chaetosphaeriales*	8290	OP023290	6	0.40%	7%	1	*Chloridium virescens* var. *caudigerum*	MH857142.1	99.81%	Vu et al. 2019
Cladosporium sp.	A	*Capnodiales*	7029	OP023229	2	0.13%	3%	1	*Cladosporium iridis*	EU167591.1	100.00%	Simon et al. 2009
Clonostachys sp.	A	*Hypocreales*	5985	OP023174	1	0.07%	2%	1	*Clonostachys rosea f. catenulata*	NR_165993.1	99.25%	Vu et al. 2019
Coniochaeta velutina FR	A	*Coniochaetales*	8334	OP023305	1	0.07%	2%	1	*Coniochaeta velutina*	MK656234.1	99.81%	van der Merwe et. al (unpublished)
Coprinellus cf. *domesticus**	B	*Agaricales*	6011	OP023184	1	0.07%	2%	1	*Coprinellus domesticus*	KP132301.1	100.00%	Irinyi et al. 2015
Coprinellus cf. *radians* 1*	B	*Agaricales*	6962	OP023221	1	0.07%	2%	1	*Coprinellus radians*	JN943117.1	100.00%	Nagy (unpublished)
Coprinellus cf. *radians* 2*	B	*Agaricales*	8590	OP023312	1	0.07%	2%	1	*Coprinellus radians*	JN943117.1	100.00%	Nagy (unpublished)
*Coprinellus disseminates**	B	*Agaricales*	5991	OP023177	12	0.79%	10%	2	*Coprinellus disseminatus*	KY977599.1	99.85%	Kranjec (unpublished)
*Coprinellus micaceus**	B	*Agaricales*	6048	OP023189	22	1.46%	19%	3	*Coprinellus micaceus*	KJ713992.1	100.00%	Jang et al. 2015
Cordycipitaceae sp.	A	*Hypocreales*	6965	OP023223	1	0.07%	2%	1	*Samsoniella hepiali*	NR_160318.1	100.00%	Wang et al. 2015
Cosmospora sp. 1	A	*Hypocreales*	7044	OP023234	1	0.07%	2%	1	*Cosmospora* sp.	MF495375.1	98.11%	Mejia et. al (unpublished)
Cosmospora sp. 2	A	*Hypocreales*	7095	OP023262	3	0.20%	5%	2	*Cosmospora lavitskiae*	KU563624.1	100.00%	Zeng and Zhuang 2016
*Cryptostroma corticale** FR	A	*Xylariales*	5932	OP023158	3	0.20%	5%	2	*Cryptostroma corticale*	MH857008.1	100.00%	Vu et al. 2019
Cyclothyriella rubronotata FR	A	*Pleosporales*	8333	OP023304	1	0.07%	2%	1	*Cyclothyriella rubronotata*	NR_147651.1	100.00%	Jaklitsch and Voglmayr 2016
Diaporthe cf. *eres*	A	*Diaporthales*	5924	OP023155	58	3.84%	43%	7	*Diaporthe eres*	MK024685.1	100.00%	Hosseini et al. 2021

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
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Diaporthe cf. *rudis*	A	*Diaporthales*	6131	OP023194	4	0.26%	7%	1	*Diaporthe rudis*	KC343234.1	100.00%	Gomes et al. 2013
Didymella sp. 1	A	*Pleosporales*	5939	OP023161	1	0.07%	2%	1	*Didymella macrostoma*	MN588154.1	100.00%	Patejuk et. al (unpublished)
Didymella sp. 2	A	*Pleosporales*	5982	OP023173	1	0.07%	2%	1	*Didymella* sp.	MW582399.1	99.78%	Wang and Pecoraro 2021
Didymella sp. 3	A	*Pleosporales*	6147	OP023193	1	0.07%	2%	1	*Didymella pinodella*	MW945409.1	100.00%	Zhao et al. 2021
Didymella sp. 4	A	*Pleosporales*	6875	OP023213	1	0.07%	2%	1	*Didymella prosopidis*	NR_137836.1	100.00%	Crous et al. 2013
Didymellaceae sp.	A	*Pleosporales*	8289	OP023289	1	0.07%	2%	1	*Didymella* sp.	MN522472.1	100.00%	Rivedal et al. 2020
Diplodia fraxini	A	*Botryosphaeriales*	5921	OP023153	280	18.49%	71%	7	*Diplodia fraxini*	MT587349.1	100.00%	Zhang et al. 2021
Diplodia sapinea FR	A	*Botryosphaeriales*	5979	OP023170	1	0.07%	2%	1	*Diplodia sapinea*	NR_152452.1	100.00%	Alves et al. 2006
Geotrichum candidum FR	A	*Saccharomycetales*	6963	OP023222	4	0.26%	3%	1	*Geotrichum candidum*	MF044044.1	100.00%	Rongfeng et al. 2018
Dothiorella sp.	A	*Botryosphaeriales*	5944	OP023164	1	0.07%	2%	1	*Dothiorella* sp.	KF040058.1	100.00%	Zlatković et al. 2016
Entoleuca sp.	A	*Xylariales*	6018	OP023187	2	0.13%	3%	1	*Entoleuca* sp.	MN538292.1	100.00%	Pusz et. al (unpublished)
Eutypa cf. *petrakii* var. *hederae*	A	*Xylariales*	5994	OP023179	5	0.33%	3%	1	*Eutypa petrakii* var. *hederae*	MT153641.1	99.82%	Bien and Damm 2020b
*Eutypa lata**	A	*Xylariales*	5937	OP023160	20	1.32%	3%	2	*Eutypa lata*	MK547093.1	99.58%	Johnston and Park (unpublished)
Exophiala sp.	A	*Chaetothyriales*	8390	OP023310	4	0.26%	7%	1	*Exophiala* sp.	LC317596.1	92.77%	Takahashi and Yaguchi (unpublished)
*Flammulina velutipes**	B	*Agaricales*	8037	OP023274	4	0.26%	2%	1	*Flammulina velutipes*	MK934583.1	99.87%	Chilean Collection of Microbial Genetic Resources (unpublished)
*Fomitopsis betulina** FR	B	*Polyporales*	8324	OP023296	1	0.07%	2%	1	*Fomitopsis betulina*	MF967582.1	99.68%	Yang et al. 2017

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
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*Fomitopsis pinicola**	B	*Polyporales*	7048	OP023253	1	0.07%	2%	1	*Fomitopsis pinicola*	JQ341137.1	100.00%	Douanla-Meli and Langer 2012
Fusarium cf. *lateritium*	A	*Hypocreales*	5919	OP023151	53	3.50%	34%	6	*Fusarium lateritium*	MN588156.1	100.00%	Patejuk et. al (unpublished)
Fusarium sambucinum	A	*Hypocreales*	6043	OP023188	1	0.07%	2%	1	*Fusarium sambucinum*	KM231813.1	100.00%	Lombard et al. 2015
Fusarium solani	A	*Hypocreales*	6003	OP023183	15	0.99%	12%	2	*Fusarium solani*	FJ459973.1	100.00%	Zhang et. al (unpublished)
Fusarium sp. 1	A	*Hypocreales*	5920	OP023152	6	0.40%	9%	2	*Fusarium iranicum*	NR_175069.1	100.00%	Sandoval-Denis (unpublished)
Fusarium sp. 2	A	*Hypocreales*	5933	OP023159	1	0.07%	2%	1	*Fusarium sporotrichioides*	MT635298.1	100.00%	Cudowski (unpublished)
Fusarium stercicola	A	*Hypocreales*	7072	OP023275	1	0.07%	2%	1	*Fusarium stercicola*	MG250476.1	100.00%	Šišić et al. 2018
Geotrichum sp.	A	*Saccharomycetales*	6955	OP023237	2	0.13%	3%	2	*Geotrichum* sp.	AY787702.2	99.10%	Lygis et al. 2005
Gliomastix sp.	A	*Hypocreales*	6957	OP023219	2	0.13%	3%	1	*Gliomastix murorum* var. *felina*	MH864097.1	100.00%	Vu et al. 2019
Graphium sp.	A	*Microascales*	8007	OP023269	1	0.07%	2%	1	*Graphium* sp.	MF782695.1	98.49%	Jankowiak et al. 2019a
*Heterobasidion annosum**	B	*Russulales*	6111	OP023195	2	0.13%	3%	1	*Heterobasidion annosum*	MH859050.1	99.66%	Vu et al. 2019
Humicolopsis cephalosporioides FR	A	*Helotiales*	7045	OP023235	1	0.07%	2%	1	*Humicolopsis cephalosporioides*	NR_160150.1	99.24%	Vu et al. 2019
Hymenoscyphus fraxineus	A	*Helotiales*	5922	OP023154	179	11.85%	53%	8	*Hymenoscyphus fraxineus*	MT155386.1	100.00%	Panteleev et. al (unpublished)
Hypholoma cf. *acutum**	B	*Agaricales*	6345	OP023209	2	0.12%	3%	2	*Hypholoma fasciculare*	MT573401.1	100.00%	Alimadadi 2019
Hypocreales sp.	A	*Hypocreales*	6930	OP023217	1	0.07%	2%	1	*Albifimbria verrucaria*	KF887115.1	99.81%	Sun et al. 2014
*Hypoxylon fragiforme**	A	*Xylariales*	6113	OP023192	4	0.26%	7%	2	*Hypoxylon fragiforme*	MH855287.1	100.00%	Vu et al. 2019

Table 2 (continued)

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*Hypoxylon howeanum**	A	*Xylariales*	7046	OP023251	1	0.07%	2%	1	*Hypoxylon howeanum*	MN588210.1	100.00%	Patejuk et. al (unpublished)
*Hypoxylon rubiginosum**	A	*Xylariales*	6051	OP023190	6	0.40%	7%	3	*Hypoxylon rubiginosum*	MZ045853.1	100.00%	Langer and Bußkamp 2021
Ilyonectria sp. 1	A	*Hypocreales*	7051	OP023252	4	0.26%	7%	2	*Ilyonectria lusitanica*	NR_156240.1	99.80%	Cabral et al. 2012
Ilyonectria sp. 2	A	*Hypocreales*	7068	OP023255	1	0.07%	2%	1	*Ilyonectria liliigena*	JF735297.1	99.80%	Cabral et al. 2012
Ilyonectria sp. 3	A	*Hypocreales*	8271	OP023281	1	0.07%	2%	1	*Ilyonectria protearum*	NR_152890.1	99.12%	Lombard et. al (unpublished)
*Jackrogersella cohaerens**	A	*Xylariales*	8292	OP023292	3	0.20%	5%	1	*Jackrogersella cohaerens*	MT561400.1	100.00%	Langer and Bußkamp (unpublished)
Jackrogersella sp.	A	*Xylariales*	6192	OP023200	4	0.26%	7%	3	*Jackrogersella* sp.	MT153658.1	99.80%	Bien and Damm 2020b
Juxtiphoma eupyrena	A	*Pleosporales*	8182	OP023280	2	0.13%	2%	1	*Juxtiphoma eupyrena*	MG098275.1	100.00%	Bußkamp et al. 2020
Kalmusia sp.	A	*Pleosporales*	7032	OP023231	1	0.07%	2%	1	*Kalmusia longispora*	JX496097.1	99.62%	Verkley et al. 2014
*Kuehneromyces mutabilis**	B	*Agaricales*	5988	OP023176	1	0.07%	2%	1	*Kuehneromyces mutabilis*	MH855190.1	99.55%	Vu et al. 2019
Lasionectria sp.	A	*Hypocreales*	6191	OP023199	1	0.07%	2%	1	*Lasionectria vulpina*	MZ159398.1	99.82%	Gaya et. al (unpublished)
Laxitextum bicolor	B	*Russulales*	8006	OP023268	1	0.07%	2%	1	*Laxitextum bicolor*	MW742677.1	99.15%	Ma and Zhao (unpublished)
Lepteutypa fuckelii	A	*Amphisphaeriales*	8284	OP023284	1	0.07%	2%	1	*Lepteutypa fuckelii*	MZ045855.1	100.00%	Langer and Bußkamp 2021
Leptodontidium sp.	A	*Helotiales*	8338	OP023309	1	0.07%	2%	1	*Leptodontidium* sp.	AB907597.1	96.72%	Fukasawa 2018
Leptosillia muelleri FR	A	*Xylariales*	6208	OP023204	2	0.13%	3%	2	*Leptosillia muelleri*	NR_164065.1	98.12%	Voglmayr et al. 2019
Lophiotrema rubi FR	A	*Pleosporales*	8327	OP023298	1	0.07%	2%	1	*Lophiotrema rubi*	AF383963.1	99.80%	Liew et al. 2002

Table 2 (continued)

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Lophiotrema sp.	A	*Pleosporales*	8337	OP023308	1	0.07%	2%	1	*Lophiotrema mucilaginosis*	NR_164039.1	100.00%	Phookamsak et al. 2019
Metapochonia bulbillosa FR	A	*Hypocreales*	6876	OP023214	2	0.13%	3%	2	*Metapochonia bulbillosa*	NR_154142.1	100.00%	Zare et al. 2000
Metapochonia suchlasporia FR	A	*Hypocreales*	8272	OP023282	1	0.07%	2%	1	*Metapochonia suchlasporia*	KU668952.1	100.00%	Palmer et al. 2018
Microcera cf. *larvarum*	A	*Hypocreales*	6195	OP023210	3	0.20%	5%	3	*Microcera larvarum*	EU860066.1	100.00%	Bills et al. 2009
Microcera rubra FR	A	*Hypocreales*	7028	OP023228	1	0.07%	2%	1	*Microcera rubra*	MH861019.1	100.00%	Vu et al. 2019
Microsphaeropsis olivacea	A	*Pleosporales*	7039	OP023250	1	0.07%	2%	1	*Microsphaeropsis olivacea*	MT561396.1	100.00%	Langer and Bußkamp (unpublished)
*Mycoacia nothofagi** FR	B	*Polyporales*	8043	OP023278	1	0.07%	2%	1	*Mycoacia nothofagi*	GU480000.1	99.83%	Moreno et al. 2011
Nectriaceae sp. 1	A	*Hypocreales*	5987	OP023175	3	0.20%	5%	2	*Cylindrocarpon* sp.	EF601608.1	99.14%	Belbahri et. al (unpublished)
Nectriaceae sp. 2	A	*Hypocreales*	8286	OP023286	2	0.13%	3%	1	*Dialonectria ullevolea*	MH855229.1	100.00%	Vu et al. 2019
*Nemania serpens**	A	*Xylariales*	6193	OP023201	6	0.40%	9%	3	*Nemania serpens*	MT790321.1	100.00%	Blumenstein et al. 2021
Neoascochyta sp.	A	*Pleosporales*	6968	OP023225	3	0.20%	5%	2	*Neoascochyta exitialis*	MT446179.1	100.00%	Liu (unpublished)
Neobulgaria sp.*	A	*Helotiales*	8331	OP023302	1	0.07%	2%	1	*Neobulgaria* sp.	MH191244.1	100.00%	Meyn et al. 2019
Neocucurbitaria acerina FR	A	*Pleosporales*	5981	OP023172	1	0.07%	2%	1	*Neocucurbitaria acerina*	MF795768.1	99.82%	Jaklitsch et al. 2018
Neocucurbitaria sp.	A	*Pleosporales*	7031	OP023230	1	0.07%	2%	1	*Neocucurbitaria cava*	KR909135.1	100.00%	Travadon et al. 2016
Neofabraea sp.	A	*Helotiales*	6125	OP023198	8	0.53%	9%	4	*Neofabraea kienholzii*	MH864120.1	100.00%	Vu et al. 2019
Neonectria punicea	A	*Hypocreales*	5980	OP023171	123	8.14%	33%	6	*Neonectria punicea*	MK955355.1	100.00%	Wu (unpublished)
Neopyrenochaeta acicola FR	A	*Pleosporales*	8328	OP023299	1	0.07%	2%	1	*Neopyrenochaeta acicola*	KJ395501.1	99.61%	Panno et al. 2013

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
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Neopyrenochaeta sp.	A	*Pleosporales*	8293	OP023293	2	0.13%	2%	1	*Neopyrenochaeta fragariae*	MT547823.1	100.00%	Bilanski et. al (unpublished)
Neosetophoma sp.	A	*Pleosporales*	5928	OP023157	1	0.07%	2%	1	*Neosetophoma rosigena*	NR_157525.1	99.64%	Wanasinghe et al. 2018
Nigrograna mycophila	A	*Pleosporales*	8330	OP023301	1	0.07%	2%	1	*Nigrograna mycophila*	NR_147654.1	99.39%	Jaklitsch and Voglmayr 2016
*Obolarina dryophila** [FR]	A	*Xylariales*	6324	OP023207	1	0.07%	2%	1	*Obolarina dryophila*	GQ428313.1	100.00%	Pažoutová et al. 2010
Oliveonia sp.	B	*Cantharellales*	8004	OP023266	1	0.07%	2%	1	*Oliveonia* sp.	MT235640.1	99.07%	Spirin et. al (unpublished)
Paracucurbitaria sp.	A	*Pleosporales*	5999	OP023181	31	2.05%	34%	6	*Paracucurbitaria corni*	MT547826.1	99.80%	Bilanski et. al (unpublished)
Paraphaeosphaeria neglecta	A	*Pleosporales*	6014	OP023186	5	0.33%	7%	3	*Paraphaeosphaeria neglecta*	NR_155629.1	99.46%	Verkley et al. 2014
Penicillium daleae [FR]	A	*Eurotiales*	6931	OP023218	2	0.13%	3%	1	*Penicillium daleae*	MH862989.1	100.00%	Vu et al. 2019
Peniophora cf. *cinerea* 1*	B	*Russulales*	6180	OP023208	5	0.33%	7%	3	*Peniophora cinerea*	MK247860.1	100.00%	Zhang (unpublished)
Peniophora cf. *cinerea* 2*	B	*Russulales*	8039	OP023277	1	0.07%	2%	1	*Peniophora cinerea*	MZ018635.1	100.00%	Volobuev and Shakhova 2022
Peniophora cf. *incarnata**	B	*Russulales*	7034	OP023249	1	0.07%	2%	1	*Peniophora incarnata*	MW740279.1	99.69%	Johnston and Park (unpublished)
*Peniophora laeta** [FR]	B	*Russulales*	8036	OP023273	1	0.07%	2%	1	*Peniophora laeta*	MH857617.1	99.33%	Vu et al. 2019
*Peniophora lycii**	B	*Russulales*	5995	OP023180	19	1.26%	10%	3	*Peniophora lycii*	MH857624.1	99.83%	Vu et al. 2019
*Peniophora quercina** [FR]	B	*Russulales*	8044	OP023279	1	0.07%	2%	1	*Peniophora quercina*	MT156129.1	99.47%	Bien and Damm 2020b
*Peniophora rufomarginata** [FR]	B	*Russulales*	7027	OP023227	1	0.07%	2%	1	*Peniophora rufomarginata*	MH857639.1	100.00%	Vu et al. 2019
Peniophora sp. 1*	B	*Russulales*	6927	OP023215	1	0.07%	2%	1	*Peniophora lycii*	MH857624.1	98.82%	Vu et al. 2019

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
									Basis of identification	ID	Identity (%)	Reference
Peniophora sp. 2*	B	*Russulales*	7226	OP023263	1	0.07%	2%	1	*Peniophora lycii*	JX046435.1	99.83%	Fedorova et. al (unpublished)
Peniophora sp. 3*	B	*Russulales*	8038	OP023276	1	0.07%	2%	1	*Peniophora lycii*	MH857624.1	97.98%	Vu et al. 2019
Pezicula cf. *sporulosa*	A	*Helotiales*	7104	OP023168	5	0.33%	3%	1	*Pezicula sporulosa*	KR859257.1	100.00%	Chen et al. 2016
Pezicula cf. *rostrupii*	A	*Helotiales*	5976	OP023245	1	0.07%	2%	1	*Pezicula rostrupii*	MH665639.1	100.00%	Marson and Hermant (unpublished)
Pezicula sp. 1	A	*Helotiales*	6112	OP023205	1	0.07%	2%	1	*Pezicula radicicola*	MH862498.1	95.05%	Vu et al. 2019
Pezicula sp. 2	A	*Helotiales*	7154	OP023260	4	0.26%	3%	2	*Pezicula* sp.	KY977580.1	96.74%	Kranjec (unpublished)
Pezicula sp. 3	A	*Helotiales*	7060	OP023240	1	0.07%	2%	1	*Pezicula ericae*	NR_155653.1	97.85%	Chen et al. 2016
Phaeosphaeria sp.	A	*Pleosporales*	7321	OP023264	1	0.07%	2%	1	*Phaeosphaeria glyceriaeplicatae*	MH862724.1	100.00%	Vu et al. 2019
Phanerochaete sordida s. lat. Gruppe*	B	*Polyporales*	7052	OP023254	1	0.07%	2%	1	*Phanerochaete sordida*	KU761238.1	100.00%	Dufresne et al. 2017
Phialocephala fortinii [FR]	A	*Helotiales*	7056	OP023238	1	0.07%	2%	1	*Phialocephala fortinii*	MT276008.1	100.00%	Myrholm and Ramsfield (unpublished)
Phialocephala piceae [FR]	A	*Helotiales*	7038	OP023216	8	0.53%	9%	2	*Phialocephala piceae*	NR_111319.1	100.00%	Schoch et al. 2014
Phialocephala sp.	A	*Helotiales*	6952	OP023236	1	0.07%	2%	1	*Phialocephala oblonga*	MG553996.1	100.00%	Haelewaters et al. 2018
*Phlebia radiata**	B	*Polyporales*	6013	OP023185	3	0.20%	5%	2	*Phlebia radiata*	MT551932.1	100.00%	Lodge et. al (unpublished)
Pleosporales sp.	A	*Pleosporales*	8335	OP023306	1	0.07%	2%	1	*Phoma herbarum*	MG586981.1	99.81%	Kirtsideli (unpublished)
Pseudeurotiaceae sp. 1	A	*Thelebolales*	6966	OP023224	1	0.07%	2%	1	*Geomyces asperulatus*	MH861038.1	100.00%	Vu et al. 2019
Pseudeurotiaceae sp. 2	A	*Thelebolales*	7152	OP023246	1	0.07%	2%	1	*Pseudogymnoascus pannorum*	MH854615.1	100.00%	Vu et al. 2019

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
									Basis of identification	ID	Identity (%)	Reference
Pseudogymnoascus sp.	A	*Thelebolales*	6432	OP023211	1	0.07%	2%	1	*Pseudogymnoascus appendiculatus*	NR_137875.1	100.00%	Malloch et al. 2016
Pseudopithomyces chartarum FR	A	*Pleosporales*	8294	OP023294	1	0.07%	2%	1	*Pseudopithomyces chartarum*	MH860227.1	99.82%	Vu et al. 2019
Ramularia sp.	A	*Mycosphaerellales*	7043	OP023233	1	0.07%	2%	1	*Ramulariacollo-cygni*	NR_154944.1	100.00%	Videira et al. 2016
Rhexocercosporidium sp.	A	*Helotiales*	7097	OP023257	1	0.07%	2%	1	*Rhexocercosporidium* sp.	MN124205.1	99.41%	Padamsee and Burgess (unpublished)
Rhizoctonia fusispora	B	*Cantharellales*	7057	OP023239	1	0.07%	2%	1	*Rhizoctonia fusispora*	MH857068.1	99.06%	Vu et al. 2019
Sarocladium cf. *dejongiae*	A	*Hypocreales*	7061	OP023241	1	0.07%	2%	1	*Sarocladium dejongiae*	NR_161153.1	99.62%	Lombard (unpublished)
*Schizopora paradoxa**	B	*Hymenochaetales*	7155	OP023261	1	0.07%	2%	1	*Schizopora paradoxa*	MH857218.1	100.00%	Vu et al. 2019
Sclerostagonospora cycadis	A	*Pleosporales*	8285	OP023285	1	0.07%	2%	1	*Sclerostagonospora cycadis*	KR611890.1	99.22%	Crous et al. 2015
*Scytalidium album** FR	A	*Helotiales*	6322	OP023206	1	0.07%	2%	1	*Scytalidium album*	NR_160102.1	99.81%	Vu et al. 2019
*Scytalidium lignicola**	A	*Helotiales*	7062	OP023242	1	0.07%	2%	1	*Scytalidium lignicola*	GU934579.1	100.00%	Bakys et. al (unpublished)
*Sistotrema oblongisporum** FR	B	*Cantharellales*	8008	OP023270	3	0.20%	3%	1	*Sistotrema oblongisporum*	KF218970.1	100.00%	Kotiranta and Larsson 2013
Sordariales sp.	A	*Sordariales*	7071	OP023256	1	0.07%	2%	1	*Podospora tetraspora*	MH859329.1	99.62%	Vu et al. 2019
Sporothrix sp.	A	*Ophiostomatales*	8291	OP023291	1	0.07%	2%	1	*Sporothrix* sp.	MH740965.1	99.62%	Jankowiak et al. 2019b
Steccherinum sp.*	B	*Polyporales*	8283	OP023283	1	0.07%	2%	1	*Steccherinum bourdotii*	MK795065.1	100.00%	Moiseenko et al. 2019
Stereum sp.*	B	*Russulales*	5970	OP023167	6	0.40%	5%	2	*Stereum armeniacum*	MH862626.1	100.00%	Vu et al. 2019
Thelonectria sp.	A	*Hypocreales*	5993	OP023178	1	0.07%	2%	1	*Thelonectria* sp.	OK161009.1	100.00%	Lutz et al. 2022

Table 2 (continued)

Species	Phylum	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results			
									Basis of identification	ID	Identity (%)	Reference
Thysanophora penicillioides FR	A	*Eurotiales*	7063	OP023243	1	0.07%	2%	1	*Thysanophora penicillioides*	MH855455.1	100.00%	Vu et al. 2019
*Trametes versicolor**	B	*Polyporales*	6137	OP023197	5	0.33%	9%	2	*Trametes versicolor*	LC710148.1	100.00%	Mori and Hirai (unpublished)
Vexillomyces sp. FR	A	*Leotiales*	7102	ON809459	1	0.07%	2%	1	*Vexillomyces verruculosus*	NR_165533.1	91.83%	Bien et al. 2020
*Xylaria longipes**	A	*Xylariales*	8288	OP023288	1	0.07%	2%	1	*Xylaria longipes*	MN588219.1	100.00%	Patejuk et. al (unpublished)
*Xylaria polymorpha**	A	*Xylariales*	5978	OP023169	7	0.46%	12%	3	*Xylaria polymorpha*	MG098262.1	99.82%	Bußkamp et al. 2020

before outgrowth could be recognised. The remaining 1070 (24%) wood chips were colonised or contaminated by yeasts, mould, or fungi which do not belong to Dikarya. The resulting pure culture isolates were assigned to 162 MT (excluding *Trichoderma* spp.) and all but ten could at least be identified to a genus level. Eighty-nine isolates could be identified to a species level (Table 2).

The majority of the isolated filamentous fungi from all samples were *Ascomycota* (132 MT including *Trichoderma* spp., 77.8%), 36 MT (22.2%) belonged to the phylum of *Basidiomycota.* Within the *Ascomycota*, the most frequently observed orders (Fig. 3) were *Hypocreales* (23.0%) followed by *Pleosporales* (22.2%), and *Helotiales* (19.8%). The *Basidiomycota* fungi were mainly represented by *Russulales* (36.1%), *Agaricales* (25.0%), and *Polyporales* (22.2%).

Despite the diversity of 162 detected fungal MT, only a few fungi occurred with high f_{MT}. Sixty-seven MT (41%) were isolated more than once and from these only 13 fungi (8%) were obtained ten or more times. The remaining 95 fungi (59%) were only isolated once. Between one and 27 different fungi were found per stem collar. On average, nine MT were recorded on each tree. None of the identified fungi were found at all sites (including extensive sampling sites) and merely 46 MT (28%) were found at more than one site. In total, 116 fungi (72%) were found solely at one site (the four subplots of Schlangen are considered one site).

Although morphologically similar to *Diplodia mutila* (Fr.) Mont., in the phylogenetic analysis based on ITS

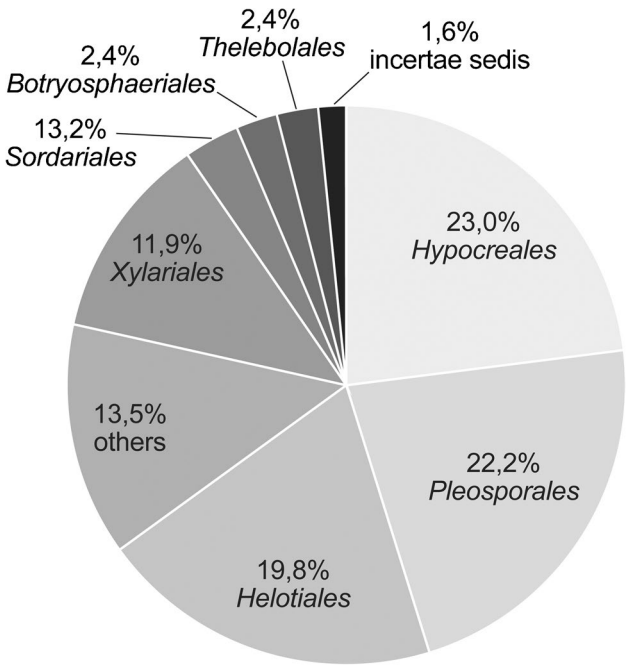

Fig. 3 Isolated orders of the *Ascomycota*, $n = 132$ of the isolated morphotypes belonging to the *Ascomycota* (Microsoft PowerPoint 2013)

sequences, the majority of *Diplodia* isolates from this study are placed clearly within a clade of strains of *Diplodia fraxini* (Fr.) Fr., including its ex-neotype (Online Resource 4 and Online Resource 6). One strain from this study (NW-FVA 5979) is placed within a clade formed by strains of *Diplodia sapinea* (Fr.) Fuckel including its ex-epitype strain and the ex-type strain of the synonymised *Diplodia intermedia*. The ITS sequences of this clade differ by one nucleotide, however, the ITS sequence of NW-FVA 5979 is identical to that of the ex-epitype strain of *D. sapinea*. Preliminary morphological observation of strains of *Armillaria* indicated affiliation to *A. gallica*. However, *A. gallica* cannot be clearly distinguished morphologically from *Armillaria cepistipes* Velen. (Tsopelas 1999). Of the 35 isolates included in the phylogenetic analysis of *Armillaria*, 33 isolates are placed within a clade containing reference strains of *A. gallica*. The two remaining isolates are placed within a clade of *A. cepistipes* (Online Resource 5 and Online Resource 7). Since only a selection of *Armillaria* isolates was included in the phylogenetic analysis due to limited lab resources, a clear distinction could not be made for all isolates of this genus. Hence, isolates of *Armillaria* are combined and referred to as *Armillaria* spp. in the final assessment of this study.

The most frequent MT isolated were *D. fraxini* (21.8%), *H. fraxineus* (13.9%), *Armillaria* spp. (12.3%), and *N. punicea* (9.6%), which account for nearly half of the isolated fungi. Perithecia of *N. punicea* were frequently observed on the ash bark above the necrotic lesions. All other isolated fungi were less frequent with <4% proportion. The most abundant MT in respect to continuity beside the aforementioned *D. fraxini* (71% continuity), *H. fraxineus* (53%), *Armillaria* spp. (50%), and *N. punicea* (33%), were *Diaporthe* cf. *eres* (43% continuity / 3.8% f_{MT}), *Fusarium* cf. *lateritium* (34% / 3.5%), and *Paracucurbitaria* sp. (34% / 2.1%). These MT were also most abundant in regard to occurrence at the nine sample sites (*H. fraxineus* occurs at eight sites, *Diaporthe* cf. *eres* and *D. fraxini* at seven sites, *Fusarium* cf. *lateritium*, *N. punicea*, and *Paracucurbitaria* sp. at six sites). When taking into account only the intensive sampling sites with at least five stem collars studied, there is an overlap of five occurring fungi: *Diaporthe* cf. *eres*, *D. fraxini*, *H. fraxineus*, *N. punicea*, and *Paracucurbitaria* sp.

The ash dieback pathogen *H. fraxineus* was isolated from 31 of the 54 stem collar necroses (57%). It was isolated at all studied sites except at Wolfenbüttel, where only a single tree with stem collar necrosis was sampled. The fungus was detected in both, young and advanced necroses, but not in the four control samples without symptomatic tissue. *Hymenoscyphus fraxineus* was isolated less frequently at the investigated sites with better water supply (soil water supply and climate combined; Online Resource 2).

147 MT (91%) were only isolated from necrotic stem tissue. Almost one-third of all MT according to their identification are able to decay wood (Table 2). A significant proportion of the isolated species were found here for the first time associated with *F. excelsior*, for example: three isolates from necrotic stem collar tissue were assigned to the MT identified as *Cryptostroma corticale* (Ellis & Everh.) P.H. Greg. & S. Waller and one isolate from stem collar tissue was identified as *D. sapinea*. The isolation of *Paracucurbitaria* sp. from the examined samples is the first proof of this genus from stem collar necroses of *F. excelsior*. Furthermore, one isolate from necrotic stem collar tissue was preliminarily assigned to the genus *Vexillomyces* S. Bien, C. Kraus & Damm based on ITS sequence comparison. Further morphologic as well as multi-locus phylogenetic investigations based on additional DNA regions (ribosomal large subunit, *EF-1α*, and a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase) revealed this isolate to represent a novel yet undescribed species of *Vexillomyces* (Tan et al. 2022). The MT, which only occurred once in asymptomatic control samples were assigned to *Akanthomyces* sp., *Cephalotrichiella penicillata* Crous, and *Sclerostagonospora cycadis* Crous & G. Okada. The following fungi were isolated from stem collar necroses as well as from symptomless controls: *Alternaria infectoria* E.G. Simmons, *Alternaria* sp., *Diaporthe* cf. *eres*, *Exophiala* sp., *Hypoxylon fragiforme* E.G. Simmons, *Jackrogersella* sp., *Paracucurbitaria* sp., *Peniophora* sp. 1 and sp. 2, *Phaeosphaeria* sp., *Pseudeurotiaceae* sp., and *Sistotrema oblongisporum* M.P. Christ. & Hauerslev.

Fungal communities in stem collar necroses

The number of MT isolated from stem collar necroses at the different sites ranged from one (Wolfenbüttel, one sample tree) to ten (Woltershausen, two sample trees) at the extensive sites and from 29 (Schwansee, eight sample trees) to 87 (Schlangen, 19 sample trees) at the intensive sites. Hence, the fungal communities at the sites studied differed in their species composition and diversity (Fig. 4). In Schlangen 52 MT (32% of all 162 isolated MT of this study) out of 87 MT were found exclusively at this site. Nevertheless, the most common MT in Schlangen are identical with the most frequent MT over all sampling sites (*Armillaria* spp., *D. fraxini*, *H. fraxineus*, and *N. punicea*). *Diplodia fraxini* was isolated from almost 80% and *H. fraxineus* from almost 70% of the trees studied at this site. 54 MT were found in the samples from the site of Berggießhübel and 30 (19%) of those occurred exclusively at this site. Likewise, the most common MT at Berggießhübel were *D. fraxini*, *H. fraxineus*, and *Armillaria* spp. However, *N. punicea* was isolated only once there. At the Rhüden study site, 32 MT were found, 13 (8%) of which were found exclusively at this site. The most frequent MT were the same as the most abundant MT in respect to continuity from all samples - except *N. punicea*,

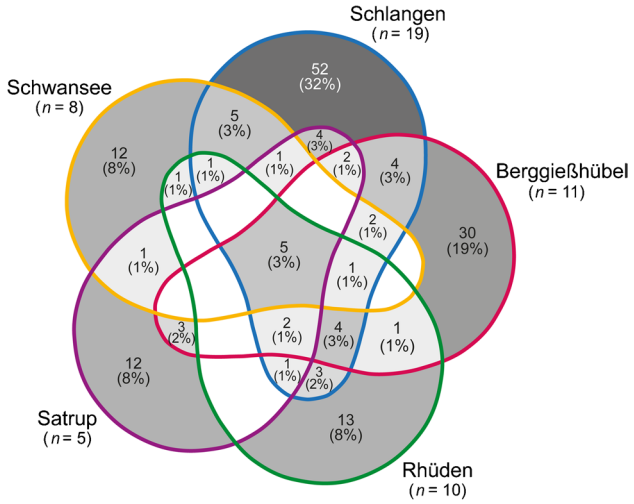

Fig. 4 Overlap between the fungi isolated from the main sites (with at least five samples) with the indication of how many of the isolated fungi were found at each site and between the different sites (absolute number as well as percantage), empty white areas indicating no overlap between the respective sites. Five fungi at the overlap of all sites are *Diaporthe* cf. *eres*, *Diplodia fraxini*, *Hymenoscyphus fraxineus*, *Neonectria punicea*, and *Paracucurbitaria* sp. (RStudio 4.1.2)

which was isolated only once in Rhüden. 31 MT were found in the Satrup samples, 12 of which (8%) exclusively at this site. The most common MT in Satrup were identical with the four most common MT in Schlangen. At Schwansee, 29 fungal MT were isolated in total, 12 of which (8%) were found exclusively at this site. At this location, the most common MT besides *D. fraxini* and *N. punicea* were *Coprinellus* species and *F. solani* s. l.

Discussion

Fungi associated with ash stem collars

In total, 162 fungi were isolated from ash stem collars and differentiated within this study. About half of these fungi were isolated from stem collar necroses in comparable studies as well (Lygis et al. 2005; Langer 2017; Meyn et al. 2019; Kranjec Orlović et al. 2020). Though 87 taxa (54%) isolated here were not reported by the aforementioned studies. It has to be taken into account that different sample sizes and sample site numbers lead to differing numbers of species. Considering the mentioned studies, Meyn et al. (2019) had the smallest sample size with four trees at one sample site and reported the smallest diversity with 16 fungal species. Langer (2017) isolated more fungal species (35) from 32 sample trees at seven sample sites. The correlation between sampling size and reported fungal diversity is also confirmed by Kranjec Orlović et al. (2020) with 68 fungal species isolated from 90 sample trees examined at three sample sites. The non-negligible impact of the number of sample sites on the detectable fungal species diversity is shown in this study with 162 MT isolated from a smaller sample size (58 trees), but a higher number of sample sites (9) compared to the study of Kranjec Orlović et al. (2020). Other factors, such as number of the incubated wood chips or the isolation method may also have influence on the number of species isolated. But overall, there seems to be a positive correlation between sample size or number of sampled sites and species richness. Langer (2017) observed that, advanced stem collar necroses result in a higher number of isolated species. This could be confirmed in the present study when taking into account only the stem collar necroses with isolation of *H. fraxineus*.

Similar to studies focusing on endophytes of tree woody tissues (Bußkamp et al. 2020; Langer et al. 2021) in this study the majority of fungi isolated belong to *Ascomycota* (77.8%). A reason for the lower frequencies of *Basidiomycota* compared to *Ascomycota* might be that fungi belonging to the former often need longer incubation periods in order to grow out from incubated woody tissues (Oses et al. 2008) but since the incubated increment segments were kept for four weeks on nutrient media, it has to be assumed that enough time was given for fungi to grow out. However, the proportion of *Basidiomycota* (22.2%) in this study is higher than in the aforementioned studies. The reason for this discrepancy might be the focus on different woody tissue types in the mentioned studies and hence detection of differing fungal communities with divergent ecological functions. *Basidiomycota* isolated from woody tissues are often related to wood rot, because lignin is primarily decomposed by this fungal group and therefore they are more likely to be found in diseased or necrotic rather than asymptomatic woody tissue (Eriksson et al. 1990; Bugg et al. 2011). Hence, the occurrence of white and brown rot fungi in stem collar necroses is not unusual. Typical white rot fungi like *Armillaria* spp., *Coprinellus* spp., *Bjerkandera adusta* (Willd.) P. Karst., *Peniophora* spp., *Trametes versicolor* (L.) Lloyd, and few brown rot fungi like *Fomitopsis* spp. have been isolated from stem collar necroses in this study. The majority of soft rot fungi isolated here pertain to *Ascomycota* and the following representatives of this group were found: *Biscogniauxia nummularia* (Bull.) Kuntze, *Hypoxylon* spp., *Jackrogersella* sp., *Nemania serpens* (Pers.) Gray, and *Xylaria* spp. (all *Xylariales*). Besides the occurrence of white and brown rot fungi, the frequent association of xylarialean wood decay fungi with stem collar necroses make it plausible that affected ash trees have a massive loss of stability and tend to topple over even without wind as a supporting factor.

Approximately one-third of the isolated MT detected in this study were listed for *F. excelsior* in the USDA fungal database (Farr and Rossman 2022). Only one of the most

frequent MT of this study, *N. punicea,* is not listed there, but other species from the genus *Neonectria* are mentioned. However, *N. punicea* was described as one of the most frequent MT associated with stem collar necroses in the context of ash dieback (Langer 2017; Meyn et al. 2019). The other most abundant MT isolated in our study *Armillaria* spp., *D. fraxini*, *H. fraxineus*, *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium,* and *Paracucurbitaria* sp. were already described to be associated with ash (Chandelier et al. 2016; Haňáčková et al. 2017; Langer 2017; Meyn et al. 2019; Linaldeddu et al. 2020; Kowalski and Bilański 2021; Barta et al. 2022). Langer (2017) investigated stem collar necroses of 32 ash trees and determined the aforementioned species as well—except for *Paracucurbitaria* sp. Meyn et al. (2019) isolated *D. fraxini* (labelled as *Botryosphaeria stevensii*), *H. fraxineus*, *N. punicea*, and *Diaporthe* cf. *eres* as well. The most commonly isolated species from stem collar necroses in the present study, except *Armillaria* sp. and *Paracucurbitaria* sp., were also isolated in high frequency by Linaldeddu et al. (2020), although they focussed on symptomatic branches of damaged ash trees in Italy. The absence of *Armillaria* sp. in branches was anticipated, because it is a soil-borne root and stem rot fungus, colonising its host through rhizomorph growth (Morrison 2004).

Within the genus *Armillaria, A. gallica* was the most frequently isolated species in the present study. Additionally to our isolations, mycelial fans and rhizomorphs of *Armillaria* spp. were observed at all sample sites of this study and the majority of further studied ash stands diseased by ash dieback. *Armillaria* species are common soil colonisers in Europe and therefore are probably existing in most forest sites even before ash dieback occurred (Morrison 2004; Lygis et al. 2005; Bakys et al. 2009b). They are considered secondary pathogens and wood-decaying fungi infecting stressed trees, which explains their occurrence in advanced stem necroses and root rot (Chandelier et al. 2016). On the one hand, *Armillaria* spp. can colonise stem collars after the necrosis has already formed by *H. fraxineus*. On the other hand, they are also able to independently attack a weakened ash tree without a stem collar necroses due to *H. fraxineus* (Langer 2017). As in our study, the occurrence of *A. gallica* and *A. cepistipes* associated with stem collar necroses of trees affected by ash dieback has been shown by Chandelier et al. (2016) in Belgium. Enderle et al. (2017) also detected *A. gallica* in stem collar rots in south-western Germany. These results are in contrast to investigations by Lygis et al. (2005), who determined *A. cepistipes* as most frequent in Lithuania. Nevertheless, regardless of which of the two species caused infection, *Armillaria* spp. most likely accelerate the decline of ash dieback-affected ash trees (Chandelier et al. 2016) and reduce stem stability.

The most frequently isolated species in our study *D. fraxini* has been recognised as the dominant species in comparable studies as well. Linaldeddu et al. (2020) determined that many reports of *D. fraxini* on ash have earlier been assigned to *D. mutila* s. l. Phylogenetic analyses showed, that most of the *Diplodia* strains isolated in this study, although morphologically similar to *D. mutila*, certainly match with *D. fraxini*. It is an aggressive pathogen known to cause bark lesions and wood discoloration or to enlarge necroses, which are primarily caused by *H. fraxineus* (Alves et al. 2014; Linaldeddu et al. 2020, 2022). Kowalski et al. (2017) classified it as the second most pathogenic fungus after *H. fraxineus,* though it was not mentioned as a frequent coloniser of *F. excelsior* before ash dieback disease occurred (Kowalski et al. 2016). These facts might indicate that infections with *H. fraxineus* facilitate the colonisation of affected ash trees by *D. fraxini*. Another possible explanation for the more frequent occurrence of *D. fraxini* could be global warming because this species benefits from warm temperatures of around 25 °C (Alves et al. 2014). In our opinion, *D. fraxini* plays an important role in ash dieback disease and contributes undoubtedly to a greater damage extent, in particular at stem collar necroses. Besides the latter very frequent *Diplodia* species, to the knowledge of the authors, this is the first report of *D. sapinea* on ash. In contrast to the study by Linaldeddu et al. (2020), the species *D. subglobosa* could not be isolated in our analysis, maybe because they investigated branches and not stem collar necroses.

Neonectria punicea has a large host spectrum, including *F. excelsior* (Hirooka et al. 2013). However, this fungus has rarely been documented from this particular host species before (Langer 2017; Meyn et al. 2019; Karadžić et al. 2020). *N. punicea* was found to be associated with stem collar necroses and cankers of European ash in Germany (Langer 2017; Meyn et al. 2019) and it is able to cause necroses in juvenile ash trees (Karadžić et al. 2020). Its perithecia were observed frequently on the bark above the necrotic ash tissue (ibid. and Karadžić et al. 2020). *Neonectria punicea* is mainly known to be a secondary pathogen, but can also express an endophytic lifestyle (Langer 2017). Species of the genus *Neonectria* invade through natural entrances, like lenticels or artificial wounds, for infection (Flack and Swinburne 1977; Salgado-Salazar and Crouch 2019).

The isolation of strains assigned to *Diaporthe* cf. *eres* were made from diseased and also from healthy woody ash tissue in this study. This is in agreement with insights that *Diaporthe eres* can live as a plant pathogen, endophyte, or saprotroph and has a wide host range as well as a widespread distribution (Udayanga et al. 2014; Linaldeddu et al. 2020). This species often produces its tiny fruit bodies on dead woody tissues (Kowalski et al. 2016). In a study by Kowalski et al. (2017), *D. eres* showed the least virulence and caused significantly milder disease symptoms on *F. excelsior* plants

than the other tested fungal species. *Diaporthe eres* could be considered a weak pathogen in comparison to ash dieback on *F. excelsior.* In case of tree weakening by *H. fraxineus* the early endophytic presence of *D. eres* favours a fast pathogenic attack (Kowalski et al. 2016).

In this study *Fusarium* cf. *lateritium* Nees has been isolated frequently from symptomatic tissue and once from healthy wood tissue. The species is already known from *F. excelsior* in association with bacterial ash canker (Riggenbach 1956) but its virulence seems to be low in comparison with other fungal species (Bakys et al. 2009b). Kowalski et al. (2017) showed, that *F. lateritium* causes none or only small necroses on *F. excelsior*. In general, *Fusarium* spp. have a wide host range and are reported as the most common endophytes in ash bark and wood (Kowalski and Kehr 1992; Sieber 2007; Bakys et al. 2009b; Kowalski et al. 2016). The facts described above, indicate that *F. lateritium* is able to colonise the bark and woody tissue of ash independently of *H. fraxineus*. In association with ash dieback though it is more likely that the species contributes to the stem collar necroses as secondary pathogen. Thereby, it is non-essential, whether acceleration of ash dieback is established by shifting from endophytic to pathogenic lifestyle or colonising the tree as a secondary pathogen after tree weakening.

As far as it is known, the isolation of *Paracucurbitaria* sp. from the examined samples is the first proof of this genus in stem collar necroses. It was not isolated by Langer (2017) and Meyn et al. (2019) from rootstock. However, Kowalski and Bilański (2021) detected *Paracucurbitaria* sp. in previous year's ash leaf petioles in Poland, Barta et al. (2022) isolated it from ash twigs in Slovakia, and Haňáčková et al. (2017) reported *Paracucurbitaria corni* (Bat. & A. F. Vital) Valenz.-Lopez, Stchigel, Guarro & Cano as an endophyte of ash leaves and seeds. Therefore the occurrence of species from the genus *Paracucurbitaria* in plant material of *F. excelsior* is not striking, but its high frequency in stem collar necroses was unanticipated. It can be assumed that the high frequency of *Paracucurbitaria sp.* is no coincidence, because its detection in stem collar necroses of ash is increasing in ongoing research at the NW-FVA since sampling for this study.

Besides *D. sapinea*, there are a few species, which, to the knowledge of the authors, have not been previously reported from *F. excelsior* (Table 2). One of them is *C. corticale*, known as the causal agent of the sooty bark disease on maples. Its main host is *Acer pseudoplatanus* L., but it has been proven that *C. corticale* can colonise other maple species as well as *Aesculus hippocastanum* L. (Enderle et al. 2020). This species was found at sampling sites with sycamore. In addition to the first reports of ash as a host, one strain belonging to the genus *Vexillomyces* was isolated and recognised as undescribed species. The genus *Vexillomyces* was described in 2020 for two species (*V. palatinus*, *V. verruculosus*) isolated from spore traps attached to vine shoots. No host organism is known for these species. Later several species of *Claussenomyces* and *Tympanis* were transferred to the genus (Baral and Quijada 2020). The respective species are known from dead or living angiosperm and gymnosperm wood, however, only for *V. atrovirens* (syn. *Claussenomyces atrovirens*) an affiliation to the host genus *Fraxinus* could be recognised (Dennis 1986).

Role of *Hymenoscyphus fraxineus* in stem collar necroses

The ash dieback pathogen *H. fraxineus* could not be isolated from all of the 54 symptomatic stem collars. Only in about half of the trees, the fungus could be determined. It has been already reported by several authors, that *H. fraxineus* could not be frequently isolated from symptomatic tissue of ash (Przybyl 2002; Bakys et al. 2009a; Enderle et al. 2017). A possible explanation for this could be its slow growth, unfavourable sampling conditions for the pathogen or too advanced necroses with antagonistic activity of other colonisers (Kowalski and Holdenrieder 2009; Hauptman et al. 2013; Gross et al. 2014; Langer 2017). Often, *H. fraxineus* could be solely isolated from recently discoloured woody tissues of the stem collar necroses (Fig. 2) and is probably supressed in the older parts of the necroses already colonised by secondary fungi. The aforementioned reasons might have contributed to the moderate isolation success of the ash dieback pathogen in this study. Perhaps, fungal community analysis by means of culture-independent methods, such as high throughput sequencing or qPCR could detect *H. fraxineus* more frequently than by culture based isolation, since these methods have the potential to detect inactively present fungi or even DNA residues if the initial fungus has been suppressed by secondary invaders (Lindahl et al. 2013). Our results on the f_{MT} and continuity of the association and localisation of *H. fraxineus* in basal stem necroses support the assumption, that this pathogen is very often the main or primary causal agent triggering stem collar necroses. Either way, *H. fraxineus* is confirmed as the main pathogenic agent of the ash dieback epidemic (Kowalski 2006; Bakys et al. 2009a; Kowalski and Holdenrieder 2009; Gross et al. 2014). The only lack of evidence for *H. fraxineus* at the study site Wolfenbüttel could be explained by the meagre sample size. It can be assumed that *H. fraxineus* may have been isolated if a larger wood chip number or sample tree size was examined. According to information from a co-researcher in FraxPath, *H. fraxineus* was present in branches of the sample trees at the study site Wolfenbüttel (Maia Ridley, personal communication).

Fungal communities in stem collar necroses

The observation of significant differences in the occurrence of fungal taxa between the investigated forest sites is consistent with the results of Bilański and Kowalski (2022). In the study of Meyn et al. (2019) only two species were found in all sample trees and many of the identified fungi were single isolates. Similarly, Kranjec Orlović et al. (2020) revealed just few predominating taxa representing half of all fungal isolates from stem bases of *Fraxinus angustifolia* Vahl. In addition, species represented only by a single isolate make up one-third of all isolates in the study by the latter authors. The fact, that the composition of fungi isolated in this study differs with only a little overlap between the sampling sites, leads to the assumption that adding further sampling sites would reveal new sets of fungi not recorded in this study.

Relation of the most common fungi to the site characteristics

Independent of age class and site conditions, European ash trees can be vulnerable to an infection by *H. fraxineus* (Pautasso et al. 2013). However, the extent of ash dieback in the crown and stem collar necroses and tree mortality, most likely depend on many different factors. Susceptibility of ash trees to the pathogen, the range of subsequent colonising fungal species (Langer et al. 2022), tree vitality, or the environmental context of the forest site and stand (Havrdová et al. 2017) are some examples. Ash tree vitality is encouraged at fertile and (moderately) wet soils, conditions which are preferred by ash (Walentowski et al. 2017). It has to be taken into account that for this study only a selection of forest sites from a rather narrow area out of the wide range of European ash was investigated. An optimal soil and water supply with a sufficient percentage of ash trees was fundamental. Furthermore, the selection of sample trees was subjected to different restrictions. For example the condition of a diameter at breast height less than 25 cm because of logistics and processing abilities in the lab. Besides that, trees with very advanced necroses like completely necrotic or rotten stem base or dead trees were not suitable for investigation.

Our preliminary results indicate that *H. fraxineus* was isolated less frequent at sites with higher water availability (Online Resource 2). This is in accordance with the guess that the fungal composition of stem collar necroses depends on soil and water availability of the forest stand (Linaldeddu et al. 2011; Salamon et al. 2020). As mentioned before, this assumption refers only to the selection of the investigated forest sites. One explanation could be, that secondary fungi have more favourable conditions at sites with higher water availability and thus are able to overgrow *H. fraxineus* faster than at drier sites.

For the other most common fungi *Armillaria* spp., *Diaporthe* cf. *eres*, *D. fraxini*, *Fusarium* cf. *lateritium*, *N. punicea*, and *Paracucurbitaria* sp., Dirichlet regression indicated no correlation between f_{WC} and water supply rank of the site (Online Resource 2). Assuming that *H. fraxineus* as the sole pathogen influences the extent of damage caused by stem collar necrosis, this this would be in contrast to the suggestion of several authors that stands with wet soil conditions show a higher probability that the individual trees affected by *H. fraxineus* exhibit greater damage (Gross et al. 2014; Erfmeier et al. 2019). At Schwansee, the wettest sampling site, *H. fraxineus* was only isolated twice. However, the stem collar necroses were most advanced at these sampling trees, where a lower isolation rate of *H. fraxineus* was generally expected, as mentioned previously.

It was noticeable, that *D. fraxini* and *N. punicea* had a significantly different f_{WC} at the various sampling sites (Online Resource 2), but there was no indication for a correlation with the site characteristics water supply, soil and bedrock, climate, or mixture of trees. However, it was observed that ash trees with a low f_{WC} of *D. fraxini* had a thinner bark. Compared with *D. fraxini* and *N. punicea*, the MT *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium*, and *Paracucurbitaria* sp. had a consistent f_{WC} over all sites. But *Fusarium* cf. *lateritium* was not isolated at Satrup and at the valley bottom in Schlangen. Due to the lower amount of isolations in this study, the authors assume there is also a lower probability of occurrence in stem collar necroses.

Armillaria species were not present at all studied sites and could not be isolated from the trees in Schwansee. This result is contradictory to those of Enderle et al. (2017), who found older necroses to be more often colonised by *Armillaria* spp. The progress of the necroses formation was clearly visible by their partially ruptured wood surface and presence of fruiting bodies on the necrotic stem areas of wood decay fungi, such as *Coprinellus* sp. and *Xylaria polymorpha* (Pers.) Grev. (Liers et al. 2011). Furthermore, the absence of *Armillaria* spp. isolates in Schwansee, the most moist of all sampling sites which is influenced by its ground water, do not correspond to preference of *Armillaria* species for continuously moist soil conditions (Whiting and Rizzo 1999). A possible explanation for the lack of this species in Schwansee, could be the specific forest site background as a former lake. The area was earlier used as fishpond until the eighteenth century. Thus, the soil was subjected to special formation conditions (Welk 2017) and perhaps it was not possible for *Armillaria* spp. to colonise the soil like in other forest sites.

Many of the other MT detected in this study were isolated just once, which may indicate no direct correlation with the investigated forest sites, thus site characteristics like soil and water supply relatedness cannot be assumed. However, it cannot be ruled out that the one-time isolated fungi occur

in other forest sites, than the investigated ones, too. As well as a higher abundancy is theoretical possible. Additionally, it is to be expected that the composition of fungi might differ according to tree age, tree species composition, forest management type, season, and the like (Scholtysik et al. 2013; Tomao et al. 2020). For example, a more diverse tree species composition at a forest site could contribute to the occurrence of a wider spectrum of fungi colonising a tree (Cavard et al. 2011; Kowalski et al. 2016; Krah et al. 2018; Tomao et al. 2020). This is confirmed by the isolation of sycamore-typical fungi like *C. corticale* und *Cy. rubronotata* from *F. excelsior* in stands with maple trees. It is furthermore supported by the fact that the most mixed intensive sampling site of Berggießhübel has one of the highest fungal diversity in relation to its sample tree amount. In addition to its diverse tree species composition, Berggießhübel is the most eastern sampling site. Satrup is the most northern sampling site and shows high fungal diversity despite its smallest sample tree amount. This observation suggests that widely varying sites in Germany lead to differing fungal communities. Furthermore, a possible underestimation of fungal diversity in the studied trees may occur since not all fungi are detectable through standardised culture based methods or in general (Guo et al. 2001; Allen et al. 2003; Unterseher 2011; Muggia et al. 2017).

Conclusion and outlook

This study provided new insights into the fungal diversity and communities of endophytes, primary and secondary pathogens, wood-decaying fungi, and saprotrophic fungi associated with stem collar necroses of European ash trees. A rich fungal composition inhabiting symptomatic stem tissue has been revealed with four frequent species occurring at most of the studied forest sites, but with little overlap between the sites. The fungal species richness detected in this study (162) is considerably higher compared to previous investigations in which 16–75 different species were detected (Lygis et al. 2005; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). This difference in diversity can be explained by the larger sampling size (not only tree number, but also amount of wood chips taken) and the partially greater number of sites studied. Single trees with only about 20 studied chips of stem collar tissue each (Oranienbaumer Heide, Wolfenbüttel) had the fewest amount of isolated MT. Further studies on stem collar necroses can increase the knowledge of fungal biodiversity on *F. excelsior*, as clearly demonstrated by the newly described species, *Vexillomyces fraxinicola* (Tan et al. 2022), which was collected in this study.

The ash dieback pathogen was isolated from only about half of the trees sampled. Different reasons like its slow growth can cause a low isolation rate of the primary pathogen. Nevertheless, it is possible that stem collar necroses are commonly initiated by this fungus. The occurrence of several pathogenic fungi from necrotic stem tissue of ash beside *H. fraxineus* is striking, because of their high f_{MT}. It was shown that the different fungal communities of the sample trees are largely dominated by three MT (*D. fraxini*, *Armillaria* spp. and *N. punicea*) next to *H. fraxineus* representing almost 50% of all isolates. They are considered to play a major role in the progression of stem collar necroses and rot and therefore also contribute to a loss of tree stability. The remaining fungi which were isolated from the stem collars necroses turned out to be very diverse with much lower f_{MT}, in the majority of cases were represented with only one isolate. Overall, the synergistic interaction of different pathogens in the context of ash dieback, for example *H. fraxineus* and *D. fraxini* or *N. punicea,* can lead to a larger damage in contrast to infection by only one pathogen (Marçais et al. 2010). In this context, *N. punicea* poses a serious threat to planted ash forests and natural regenerations of *F. excelsior*, especially if another host tree species, such as European beech (*Fagus sylvatica* L.) is in mixture. European beech is potentially an inoculum reservoir of *N. punicea* for future infections of ash stem collars (Karadžić et al. 2020). Therefore, in the future, the susceptibility of ash to form stem collar necroses and to be diseased by *D. fraxini* and *N. punicea* should be considered in breeding programmes to develop more resistant ash trees in relation to ash dieback.

However, stem collar necrosis types caused by other fungi than *H. fraxineus* or *Phytophthora* spp. (Langer 2017), should not be disregarded. The results of this study show, that at least one fungal pathogen can be found in the necrosis without evidence of *H. fraxineus.* For example, one of the control samples, which turned out to have necrotic tissue inside the wooden body, was colonised by *Armillaria* sp. In this case, it is likely that the fungus attacked the weakened tree independently of a pre-colonisation of the stem collar by *H. fraxineus*.

Since in this study no correlation between the site factors and fungal occurrence could be calculated because most of the isolated fungi were only detected once, further studies should be carried out at additional comparable forest sites. Inventories of stem collar necroses at a higher number of locations may reveal dependence of MT to forest side conditions and their individual role in the fungal communities in detail. Future studies need to be conducted in order to estimate potentially high-risk characteristics of forest sites for pronounced and fast-advancing stem collar necroses and rot. Additionally, the investigation of genotypes of *H. fraxineus* associated with single-stem collar necroses could help to better understand the path of infection with *H. fraxineus* and the secondary colonisation by other fungi.

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Data availability The DNA sequences generated in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov; Table 2). All sampling data is provided in the online resources (ESM 3). The fungal strains are stored in the strain collection of the NW-FVA. Text and images are permanently stored on an internal drive of the NW-FVA.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

Conflict of interest The authors declare no competing interests.

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3. Manuscript II

Peters S, Gruschwitz N, Bien S, Fuchs S, Bubner B, Blunk V, Langer GJ, Langer EJ (2024) The fungal predominance in stem collar necroses of *Fraxinus excelsior* – a study on *Hymenoscyphus fraxineus* multilocus genotypes. J Plant Dis Prot. https://doi.org/10.1007/s41348-024-00912-2

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The fungal predominance in stem collar necroses of *Fraxinus excelsior*: a study on *Hymenoscyphus fraxineus* multilocus genotypes

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Abstract
Over the past decades, European ash trees in Germany have been affected by ash dieback, reducing their vigour and mechanical resistance. Those trees that also have stem collar necroses and the resulting stem rot are particularly affected. In this study, multilocus genotypes (MLGs) of *Hymenoscyphus fraxineus* and their interactions with other fungi from stem collar necroses were analysed. Ten ash trees from three different adjacent forest stands in central Germany were sampled. A total number of 716 isolates were obtained from stem collar necroses from these ten trees. Microsatellite analysis was successfully performed on 274 isolates identified as *H. fraxineus* and 26 MLGs were revealed. The number of MLGs varied from one to seven per tree and did not correspond to the number or severity of necroses. A striking result was that five of the MLGs occurred in two trees. All other MLGs occurred independently in only one tree, as expected. Our data show that when multiple MLGs were observed in a tree, one of the MLGs outnumbered the others, indicating that *H. fraxineus* is a primary coloniser of stem collar necroses. A total of 61 morphotypes, including *H. fraxineus*, were identified and discussed, comprising endophytic, saprotrophic and pathogenic fungi. Between five and 19 different fungi were found per stem collar necrosis. The majority of all isolated morphotypes were *Ascomycota* (82%), with the most common orders being *Xylariales* and *Hypocreales*. The most frequently isolated morphotypes, apart from *H. fraxineus*, were *Armillaria* sp. and *Diplodia fraxini*. Together they account for more than three quarters of all assigned isolations. Apart from *H. fraxineus*, only *Diplodia fraxini* was isolated from all ten trees.

Keywords *Hymenoscyphus fraxineus* · *Fraxinus excelsior* · Multilocus genotypes · Ash dieback · Stem collar necroses

Introduction

The invasive fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (*Helotiaceae*, *Ascomycota*) is the cause of ash dieback, a severe disease that has affected European ash (*Fraxinus excelsior* L.; hereafter referred to as ash trees) since the early 1990s, initially in Poland and Lithuania, and is now widespread in Europe (Przybyl 2002; Lygis et al. 2005). The first confirmation of the pathogen in Germany was in 2006, but the disease has probably been present here since 2002 (Heydeck et al. 2005; Schumacher et al. 2007). Ash trees of all ages are infected with many different symptoms, including stem collar damage such as stem collar and root necrosis, or crown damage such as wilting, sunken cankers, leaf necrosis, inner bark discoloration, shoot blight or epicormic shoots (Gross et al. 2014a; Langer 2017). In terms of stem collar damage, stem collar necrosis (SCN) is a lesion on the stem consisting of necrotic tissue on the outside and inside. The form of the SCN is influenced by the fungi involved, the progression of the SCN according to the conditions present, or other factors. When the process is already more advanced, the initial infection by *H. fraxineus* is followed by other fungi that can cause wood rot at the

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base of the stem. The composition of these SCN associated fungal communities has recently been investigated in initial studies by Peters et al. (2023). As the fungus caused SCN spreads to the xylem and heartwood of ash trees, it reduces their stability and increases mortality (Langer 2017). Some of the fungi associated with SCN are able to decay wood. Wood-decaying fungi can be classified into three major groups based on their ability to degrade lignin and the type of wood decay: white rot fungi, brown rot fungi and soft rot fungi (Blanchette 1991; Langer et al. 2021). Through the production of ligninolytic extracellular oxidative enzymes, white rot fungi (*Basidiomycota*) are able to degrade lignin most effectively. The lignin removed from the wood reduces the stability of the tree and can cause the ash to fall (Linhares et al. 2021). A common white rot fungus on ash is, for example, *Armillaria* spp. (Sahu et al. 2021).

Even if the crown of an ash tree appears almost vital and healthy, SCN may be present on the trunk. However, they are more often found on severely damaged trees (Schumacher et al. 2010; Husson et al. 2012; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). *Hymenoscyphus fraxineus* has a heterothallic mating system and produces asexual conidia in its anamorphic stage. The development and biology of the pathogen are described in detail in Gross et al. (2012b). Infection of ash by *H. fraxineus* is primarily caused by ascospores, which can be dispersed by wind over long distances. So far, there is no evidence that the asexually formed, sticky and non-infectious conidia of the anamorph play a significant role in the spread. They are thought to serve as spermatia during sexual reproduction (Gross et al. 2012b; Kirisits et al. 2013). However, there is also contrary information that *H. fraxineus* conidia may have an infectious effect (Fones et al. 2016).

Previous microsatellite analyses of *H. fraxineus* have already shown that there is a high genotypic diversity (Gross et al. 2014b; Nguyen et al. 2016) and that different multilocus genotypes (MLGs) can occur in ash shoots, branches and stems (Bengtsson et al. 2014; Meyn et al. 2019). Multiple infections by the pathogen are the most likely cause of different MLGs found in the same plant. There is no genetic differentiation between genotypes that infect the tree via the crown and those that attack at other compartments of the respective tree (e.g. at the stem base), and therefore all *H. fraxineus* MLGs seem to have the potential for stem collar infection (Burokiene et al. 2015).

This work is based on the hypothesis that one *H. fraxineus* individual per SCN indicates that the pathogen is a primary coloniser, biotrophically invading plant tissues. The presence of multiple *H. fraxineus* individuals per SCN would indicate a saprophytic lifestyle, with many individuals colonising pre-damaged tissue due to high spore pressure or a succession of multiple infections over time. This study is part of the FraxForFuture demonstration project and the FraxPath and FraxGen sub-networks (Langer et al. 2022). The aims of this research are to investigate the following questions: (i) How many *H. fraxineus* MLGs are involved in causing SCN in the ash trees studied? (ii) If the same *H. fraxineus* MLGs are present in SCN of different trees, are they different individuals? (iii) Is there a correlation between *H. fraxineus* MLGs and the type of SCN or other fungi associated with the SCN?

Materials and methods

Sampling site and stand

The sampling site selected for this study is not part of the common sampling site set in FraxForFuture (Langer et al. 2022). The three sampling plots are in close proximity (< 500 m distance) and are located in a mixed broadleaved forest site in Holzminden (Germany, Lower Saxony; hereafter referred to as forest site Holzminden; Fig. 1). The sampling plots have a comparable stand structure and a high proportion of ash trees. According to the forest management plan, and counting the annual rings of the sampled trees, these sampling plots are composed of trees that are approximately 35–45 years old. Plots I and II had a very similar proportion of ash (about 25%) and an ash tree density of 366 and 362 trees/ha, respectively. Plot III had a higher proportion (about 55%) and density (768 trees/ha) of ash trees. Other tree species mixed with *F. excelsior* were mainly *Fagus sylvatica* L., *Acer pseudoplatanus* L., *Prunus avium* L., *Ulmus glabra* Huds. and *Tilia platyphyllos* Scop. in decreasing proportions.

The forest site Holzminden, with its three sampling plots, extends along a relatively steep (15–25°) western slope of a north–south oriented hill ridge in the southern part of Lower Saxony, within the Weser uplands. The slope is covered by a solifluction layer of Triassic limestone over claystone and sandstone of the upper bunter formation. Both substrates are nutrient-rich (calcareous-eutrophic), and well drained due to the steepness of the slope and the high coarse fraction in the solifluction cover layer. Therefore, the soil water regime of the plot I and plot II is relatively dry. Plot III is slightly moister than the other two plots due to its lower position on the slope and the higher proportion of clayey loam in the soil.

All ash trees on the plots were inspected and the stem base and the crown condition were classified following the guidelines of the ash dieback classification key (Peters et al. 2021a, 2021b) with slight modifications. The crown condition was rated on a scale of six damage classes (Fig. 1, in line with the mentioned key). The stem base was investigated slightly differently from the key, resulting in four damage classes depending on the proportion of affected surface.

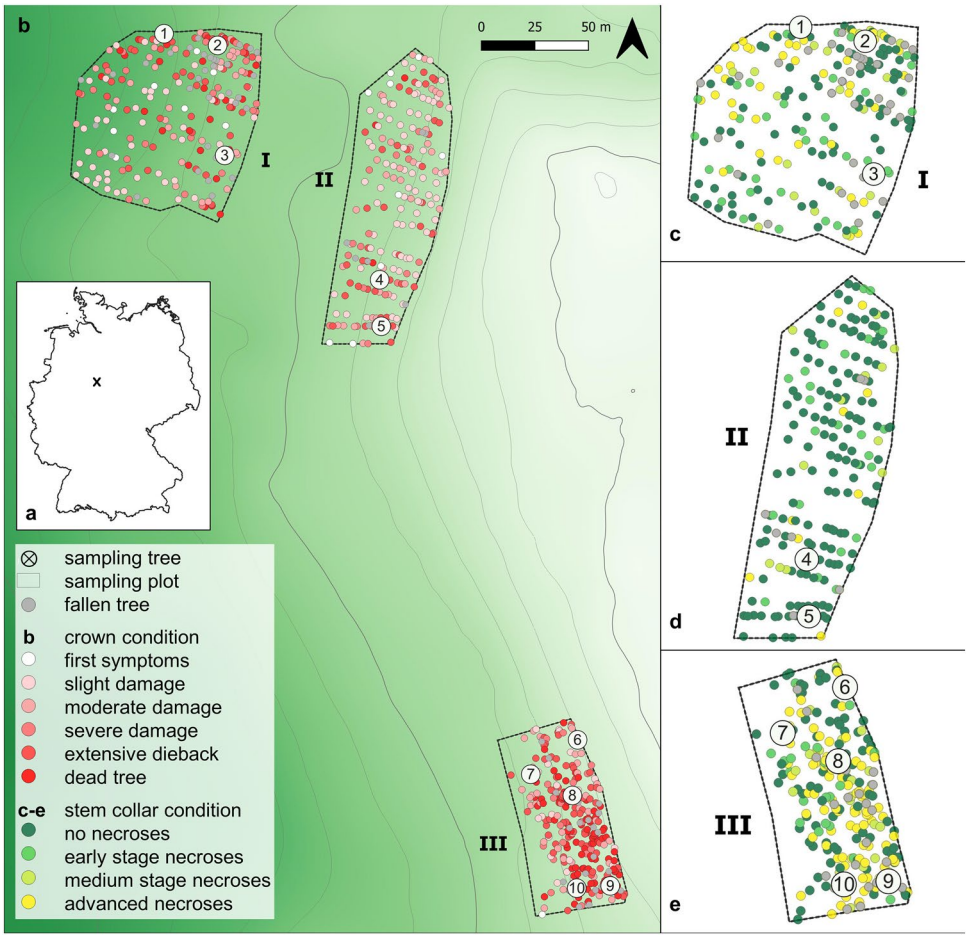

Fig. 1 Location of the forest site Holzminden in Germany marked with an x (**a**). Consisting of three sampling plots and ten sampled trees. Crown condition of all ash trees in the sampling plots (**b**) and an enlarged view of sampling plots I, II and III with condition of the stem base (**c-e**). © GeoBasis-DE/BKG (2021); extract from the geodata of the Landesamt für Geoinformation und Landesvermessung Niedersachsen, © 2020 (QGIS Desktop 3.24.3)

Sampled trees

A total of ten ash trees (Online Resource 1) with a diameter at breast height between 10 and 16 cm were sampled in the forest site Holzminden. Trees 1, 2 and 3 were taken from sampling plot I, trees 4 and 5 from sampling plot II, and trees 6 to 10 from the southernmost sampling plot III (Fig. 1).

The ash trees were felled in March 2022 and cut at least 15 cm above the visible necrotic area at the stem base. The trunk bases and the uppermost parts of the main roots were then dug out with picks and shovels. Stem collars were transported to the laboratory in clean and labelled plastic bags.

Isolation and identification of fungi

In order to identify *Dikarya* Hibbett, T. Y. James & Vilgalys associated with SCN and to determine the fungal community, a culture-based method was chosen, including isolation of fungi from wood chips and identification of the resulting fungal species based on morphological observation and molecular analysis. The procedures for wood sample preparation, fungal culturing on malt yeast peptone (MYP; Langer 1994) agar medium, DNA extraction, PCR and sequencing of the ITS DNA region, subsequent morphotype (MT) designation and identification based on observed morphology and BLASTN results were carried out according to Peters et al. (2023). From each of the ten trees, three additional wood chips of asymptomatic stem tissue were taken as control samples. Fungi that could not be isolated because they were overgrown by other fungi or were contaminated, were grouped together as "Fungus sp.". Yeasts and obvious plate contaminations were not considered. For each MT, at least one representative culture was placed in storage on MYP slant tubes at 4 °C in the fungal culture collection of the Northwest German Forest Research Institute (NW-FVA). Generated sequences were submitted to GenBank (Online Resource 2; http://www.ncbi.nlm.nih.gov/genbank; Altschul et al. 1997).

The frequency of each isolated fungal MT among all isolates was given as the percentage of that particular MT compared to all isolates. The continuity of isolated MTs was defined as the number of sampled trees in which the MT was detected in relation to the total number of sampled trees (Online Resource 2).

The relative abundances of the most common fungi relationships were evaluated visually using scatterplots. Relationships between these fungi and the number of MLGs were also evaluated. A possible relationship between the number of MLGs or the most common fungi and the fungal diversity was also analysed by comparing relative abundances. Trees were subject to varying sampling efforts with respect to fungal alpha diversity, making observed fungal alpha diversity numbers an outcome of a function of sample size (wood chips) per tree. Individual-based rarefaction (Gotelli and Colwell 2001) of samples from all other trees to the same number of samples is based on the principle: What fungal alpha diversity number would have been reported for each of the trees if all trees had the same sample size as the tree with the fewest samples? Rarefaction analysis (Sanders 1968) is methodologically based on a random subset of samples per tree and therefore needs to be repeated a sufficient number of times, here 1000, to fully exploit the tree-specific empirical sample. In each of the randomly generated sub-samples, the unique number of MTs is counted and divided by the total number of all unique MTs in all samples. The mean of these 1000 values is reported for each tree.

Microsatellite genotyping of *Hymenoscyphus fraxineus*

Microsatellite genotyping was performed on isolates that were morphologically identified as *H. fraxineus*. Fungal material was harvested by scraping fresh aerial mycelium from *H. fraxineus* cultures on agar plates. Homogenisation was performed in a Biolab Omni Bead Ruptor 24. (Biolab, Bebensee, Germany) for 2 × 30 s using five 1.4 mm ceramic beads (Omni International, Georgia, USA). *H. fraxineus* DNA was extracted using a Qiagen DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 20 microsatellite loci were amplified in a multiplex PCR, using the Qiagen Type-it ® Microsatellite PCR Kit (Qiagen) and fluorescence-labelled primers (Table 1). The primers were produced by biomers.net GmbH (biomers, Ulm, Germany). Fragments were amplified in a total reaction volume of 12.5 µl using 1 µl DNA template (5 ng/µl), 6.25 µl PCR multiplex mastermix, 2.75 µl distilled water 1.25 µl Q solution, and 1.25 µl primer mix (Table 1).

The PCR was performed in a Peqlab Thermocycler (Peqlab, Erlangen, Germany) with an initial denaturation step of 15 min followed by 30 cycles of 30 s denaturation at 95 °C, 90 s annealing at 56 °C and 60 s elongation at 72 °C. This was followed by a final elongation of 30 min at 60 °C. Fragment length analysis of the PCR product was performed in a GeXP DNA analyser (Beckman Coulter, Brea, USA). Fragment length determination and allele assignment were performed using the GeXP fragment analysis tool (Beckman Coulter). After checking each sample individually, an allele table was manually created in Excel.

Table 1 Content of the three primer mixes used for microsatellite PCR

Mix	Locus	Motif	References	Final concentration (µM)	Fluorescent dye
1	Chafra01	$(AG)_{15}(TG)_{11}$	Bengtsson et al. (2012)	0.4	DY-682
	Chafra02	$(AG)_{15}(TG)_{11}$	Bengtsson et al. (2012)	2	Cy5
	Chafra03	$(AG)_{21}$	Bengtsson et al. (2012)	2	DY-751
	Chafra04	$(TG)_{16}TC(GT)_4$	Bengtsson et al. (2012)	2	DY-682
	Chafra09	$(GCA)_8$	Bengtsson et al. (2012)	2	DY-751
	Chafra14	$(AG)_2AT(AG)_{12}$	Bengtsson et al. (2012)	2	Cy5
	SSR114	$(T)_{11}$	Gherghel et al. (2014)	2	Cy5
2	mHp_060142	$(GAA)_7$	Gross et al. (2012a)	2	DY-751
	mHp_080495	$(CT)_{10}$	Gross et al. (2012a)	2	DY-682
	mHp_088853	$(GGT)_7$	Gross et al. (2012a)	4	DY-751
	mHp_092622	$(CTC)_{10}$	Gross et al. (2012a)	2	Cy5
	mHp_095478	$(TA)_9$	Gross et al. (2012a)	2	Cy5
	mHp_103438	$(ACC)_8$	Gross et al. (2012a)	2	DY-682
3	mHp_080497	$(TCG)_8$	Gross et al. (2012a)	4	DY-751
	mHp_066169	$(GGAT)_5$	Gross et al. (2012a)	4	DY-682
	mHp_079915	$(ACC)_7$	Gross et al. (2012a)	2	DY-682
	mHP_067022	$(TCA)_8$	Gross et al. (2012a)	2	Cy5
	mHp_077098	$(AGT)_8$	Gross et al. (2012a)	2	DY-751
	mHp_095481	$(TTCC)_6$	Gross et al. (2012a)	2	Cy5
	Chafra 13	$(TC)_{13}$	Bengtsson et al. (2012)	2	Cy5

Statistical analysis of microsatellite data

The Excel add-in GeneAlEx 6.2 was used to determine the number of different MLGs present in one sampled tree, considering each tree as a unique population. The probability P_G that two or more individuals in a dataset are similar by chance was calculated for each MLG according to Milgroom (2015) (Online Resource 3), using the product of the allele frequencies for each respective locus.

Re-analysis of published crossing data

Published data from Gross et al. (2012b) was re-analysed to identify identical MLGs in the dataset of the crossing experiments. Gross et al. (2012b) were able to perform a cross between the strains Münc_r_41 and Münc_r_38 (Crossing A in Fig. 2) and a second cross between the strains Abts_22 and Abts_33 (Crossing B in Fig. 2), which resulted in apothecia with viable ascospores (Table 3 in Gross et al. 2012b). They prepared single-spore cultures from ascospores of the two crossings. In the original publication, allelic data from the offspring was used to confirm that the alleles of both parents were present in the 96 progeny single-spore cultures tested. The frequency of MLGs was not relevant in the study of Gross et al. (2012b).

The aim of the re-analysis was to determine the potential of the microsatellite assay used in this study to differentiate closely related individuals. The similarity of the datasets (Online Resource 4) should allow conclusions to be drawn about the resolution of the primer assay of Gross et al. (2012b) and the one used in this study. The original allele table was kindly provided by Andrin Gross. The Excel add-in GenAlEx was used to determine the number of MLGs in the two full sibling populations.

Results

Sampling site and stand

A total of 703 ash trees were assessed to select ten sampled trees for analysis in this study. Almost 10% of all ash trees had already fallen, probably due to a combination of natural thinning and ash dieback. The rate of fallen ash trees was highest on plot III (with the highest proportion and density of ash) and lowest on plot II. Forty per cent of all ash trees had severe crown damage or were already dead (standing). More than half of these severely damaged ash trees were located on sampling plot III. Only five trees in all sampling plots showed very few symptoms of ash dieback in the crown. However, 41% of the trees, mainly located on sampling plot II, showed no damage at the base of the stem. The most severe SCN were mainly found on sampling plots I and III. Sampling plot III showed the most severe damage in the crown and at the base of the stem, followed by sampling plot I. Sampling plot II showed the least damage in the crown and at the base of the stem (Online Resource 5).

Sampled trees

The crown condition of the sampled trees ranged from slightly damaged to severely damaged (crown level 2 to 3 according to Peters et al. (2021a)). All trees had at least one obvious SCN with discoloured, sunken, and in some cases ruptured bark. The number of SCN per tree, based on the externally visible tops of the necrotic areas, varied from one to five. For seven out of ten sampled trees, more than half of the trunk was necrotic. Tree 6 had the lowest necrotic stem circumference with 25% of the stem being necrotic, while tree 7 had the highest with 90%. The ratio of the height of the examined SCN to the diameter of the stem base was on average two and a half times the diameter of the stem base. In four out of ten cases, a ruptured wood surface was visible at the SCN. With the exception of tree 1, white rot was visible in all trees (Table 2).

Isolated fungi

A total of 1497 wood chips of stem collar tissue originating from ten ash trees were incubated. Overall, 716 mycelial outgrowths were observed on 676 wood chips (45% of all chips incubated). Thirty outgrowths were unidentifiable due to contamination and were classified as "Fungus sp.", giving

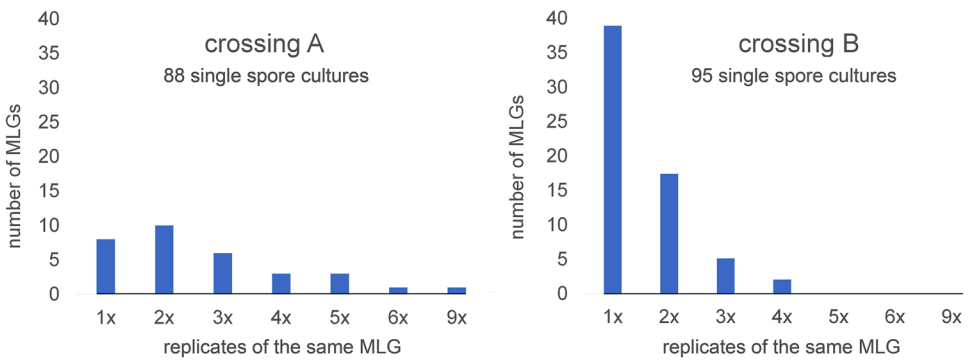

Fig. 2 Frequency of the occurrence of multilocus genotypes (MLGs) in the offspring of two crossings of *Hymenoscyphus fraxineus* individuals in a published crossing experiment (Gross et al. 2012b). Crossing A: strain Münc_r_38 × strain Münc_r_41, crossing B: strain Abts_22 × strain Abts_33

Table 2 Overview of the ten sampled trees with information about their stem collar necroses (SCN) and the number of additionally isolated fungi. The SCN classification was conducted according to Peters et al. (2021a). The number of SCN is related to the externally visible top ends of necrotic areas. The height of the SCN is related to the diameter of the stem base. For trees with more than one SCN, the highest one was selected. The number of additionally isolated fungi includes three endophytes from symptomless ash stem tissue

Sampled tree	Sampling plot	Stem collar necroses						Number of additionally isolated fungi
		Classification	Number	Height	Circumference (%)	Ruptured wood surface	Visible white rot	
1	I	2	3	2.5	60	No	No	4
2	I	2	3	2	70	No	Yes	7
3	I	2	1	2	35	No	Yes	15
4	II	2	2	3.5	80	No	Yes	9
5	II	2	2	2.5	50	Yes	Yes	11
6	III	2	2	3	25	Yes	Yes	8
7	III	2	5	2.5	90	Yes	Yes	18
8	III	2	3	2	75	Yes	Yes	15
9	III	1	3	1	65	No	Yes	15
10	III	2	2	5	40	No	Yes	9

a total of 686 fungal strains. Altogether, 377 chips (25%) showed no outgrowth at all after four weeks of incubation, while 278 (19%) chips had been overgrown by fast-growing fungi from adjacent wood chips before outgrowth could be detected. The remaining 166 (11%) wood chips were colonised or contaminated by yeasts, moulds, *Trichoderma* spp. or non-dikarya fungi. The resulting 686 fungal strains were assigned to 61 MTs (excluding *Trichoderma* spp.), comprising endophytic, saprotrophic and pathogenic fungi. Thirty-two MTs could be identified to species level. Twenty-two isolates could be identified to at least genus level (Online Resource 2). The majority of all isolated MTs were *Ascomycota* (50 MTs, 82%), 11 MTs (18%) belonged to the division of *Basidiomycota*. Within the *Ascomycota*, the most common orders were *Xylariales* and *Hypocreales* (both 22%), followed by *Pleosporales* (14%), and *Helotiales* (12%). The *Basidiomycota* fungi were mainly represented by *Russulales*, *Agaricales* and *Polyporales* (three species each).

Despite the diversity of the 61 fungal MTs detected, only five fungi had a frequency of more than 2%. The most frequent isolated MTs (Online Resource 2) were *H. fraxineus* (40.5%), followed by *Armillaria* sp. (22%), *Diplodia fraxini* (Fr.) Fr. (15.2%), *Diaporthe* cf. *eres* (3.1%) and *Paracucurbitaria* sp. (2.5%). *Diplodia fraxini* and *H. fraxineus* were the most continuous MTs, occurring on all studied trees (100%), followed by *Armillaria* sp. (90%). Together, the latter three MTs already accounted for more than three quarters of all assigned isolations. In at least half of the trees, the MTs *Paracucurbitaria* sp., *Fusarium* cf. *lateritium* and *Angustimassarina* sp. were also present. Furthermore, the MTs *Angustimassarina* sp., *Armillaria* sp., *Diaporthe* cf. *eres*, *D. fraxini*, *Fusarium* cf. *lateritium*, *H. fraxineus* and *Paracucurbitaria* sp. were isolated from all three sampling plots.

Between five and 19 different fungi, including *H. fraxineus*, were found per SCN. On average, 12 MTs were recorded per tree. Forty-two of the 61 MTs were isolated from a single tree. The MTs *Cytospora* sp., *Hypocreales* sp., and *Vuilleminia* sp. were only isolated from asymptomatic tissue.

The graphical comparison of the relative abundances of the most common fungi *Armillaria* spp., *D. fraxini* and *H. fraxineus* with each other and with the MLG numbers, as well as the most common fungi and the MLG numbers with the fungal diversity showed no clear evidence of underlying strong dependencies (figures not shown).

Population genetics of *Hymenoscyphus fraxineus*

Genetic diversity

The multiplex primers identified 26 different MLGs (A-Z) in the set of 274 *H. fraxineus* samples derived from ten sampled trees with different numbers of SCN (Online Resource 6), indicating a low genotypic diversity. The loci Chafra02 and mHp_077098 were monomorphic and therefore excluded from further analysis. Allelic diversity was low, with the majority of loci (16/18) being biallelic. The locus mHp_088853 showed a third allele in two isolations from sampled tree 9. The locus Chafra03 had a maximum of five alleles, with the most frequent alleles being 210 and 190. The other three alleles occurred exclusively in sampled trees 6 and 7. While the alleles 212 and 214 occurred with a high frequency, allele 229 occurred only once.

Nine *H. fraxineus* MLGs were found in sampling plot I, two in sampling plot II and 15 in sampling plot III. Considering the different number of sampled trees in each sampling plot, the proportion of MLGs per sampling plot

was three (I), one (II) and three (III). The largest number of *H. fraxineus* MLGs (7) was found in sampling plot III, where the highest number of samples was taken because the crown and stem collar damages due to ash dieback (Fig. 1) were most severe.

Probability of identity

To determine the probability of two or more individuals in a dataset being similar by chance, the probability of identity P_G was calculated. The calculated P_G values ranged from 10^{-4} to 10^{-7} (Online Resource 3), which suggests that in the considered dataset of 274 samples it is unlikely to find two MLGs that are identical by chance, assuming that the analysed individuals are unrelated.

Re-analysis of published data

To assess the resolution power of the primer assay used in this study, published data from Gross et al. (2012b) of two crossing experiments of single-spore cultures was re-analysed. The majority (12/20) of the primers used in this study were also used in Gross et al. (2012b) (Online Resource 4), allowing conclusions to be drawn about the resolution power of the primer assay. Furthermore, the total number of primers used in both studies was similar.

Due to some missing data in the original allelic tables of 2 × 96 single-spore cultures, re-analysis was possible for 88 (crossing A) and 95 (crossing B) single-spore cultures. The re-analysis showed the occurrence of identical MLGs within both of the crossing experiments (Fig. 2). Among the 88 descendants of the Münc_r_41 × Münc_r_38 crossing, only eight unique MLGs could be identified. Every other MLG occurred at least twice and a maximum of nine times. In the Abts_22 × Abts_33 crossing, 38 of the 95 descendants had unique MLGs. This suggests that a set of 19 primers representing 17 nuclear polymorphic loci cannot reliably distinguish between full sibling individuals. Therefore, the microsatellite array used in this study, with 20 primers representing 18 nuclear polymorphic loci, is also unlikely to reliably distinguish between closely related individuals.

Number of multilocus genotypes per sampled tree

In order to assess the genetic diversity within a tree, the number of individuals per sampled tree was determined, whereas each individual is represented by one MLG. In sampled trees 4, 5 and 8, only a single MLG was present. In trees with multiple MLGs, one MLG outnumbered the others with a frequency of more than 50% (Fig. 3, Online Resource 6). This was the case for all sampled trees except 7 and 9. A maximum of seven MLGs could be identified in sampled tree 7, without one MLG dominating the others (Fig. 3). This was also the tree with the largest number of samples.

Five (L, M, N, O and P) of the seven MLGs occurring in sampled tree 7 were also isolated from sampled tree 6. These two trees grew relatively close to each other compared to the possible pairwise distances of all sampled trees analysed (Fig. 4).

The frequency of occurrence of the identical MLGs in sampled tree 6 and 7 was different in both trees (Fig. 3). While L was the most frequently isolated MLG in sampled tree 6, sampled tree 7 was dominated by MLG O. Some of the shared MLGs (M, N and O) between sampled tree 6 and sampled tree 7 had alleles (212, 214) at locus Chafra03 that are unique in the dataset (Online Resource 6).

Number of multilocus genotypes per stem collar necrosis

The aim of this study was to determine the number of *H. fraxineus* MLGs present in a single SCN. As the SCN of trees with multiple infections tended to overlap, a distinct

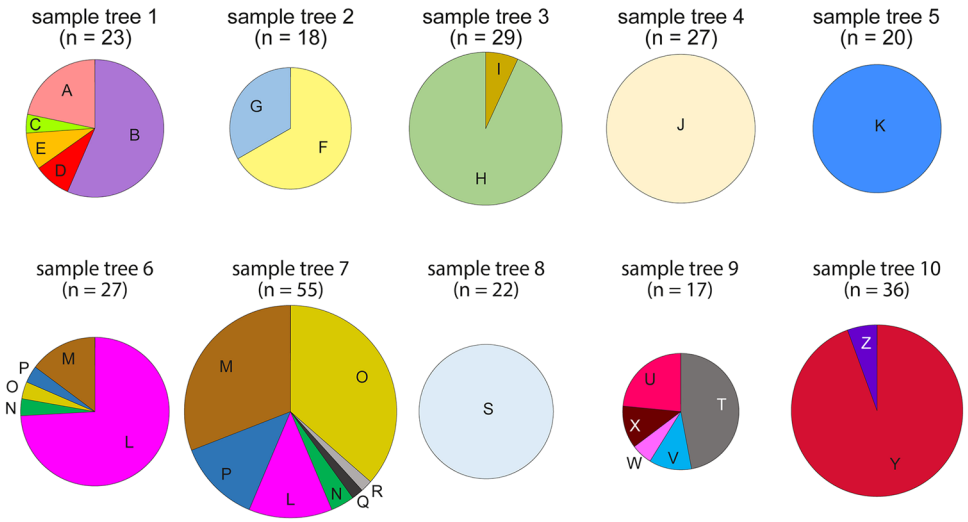

Fig. 3 Relative abundance of multilocus genotypes (A-Z) per sampled tree. The size of the pie charts refers to the respective sample size (n). Trees 4, 5 and 8 had one, trees 2, 3 and 10 two, trees 1, 6 and 9 five and tree 7 seven confirmed infections with *Hymenoscyphus fraxineus*

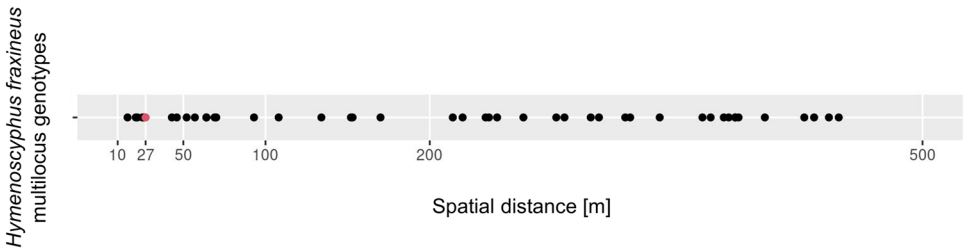

Fig. 4 Spatial distances of all sampled tree pairs (dots) in the forest site Holzminden. The distance between the sampled trees 6 and 7 is 27 m. This is the only tree pair with identical *Hymenoscyphus fraxineus* multilocus genotypes (red dot)

spatial assignment was often not possible. In no tree, except for sampled tree 10, did the number of detected SCN correspond to the number of MLGs present (Table 2, Online Resource 6). Sampled tree 7 had the highest number of MLGs (7) and at the same time the most visible SCN (5), but it was also evaluated with the second highest number of incubated wood chips (Table 2, Fig. 3, Online Resource 6).

Furthermore, the number of MLGs seems to be independent of the size of the SCN, as both the lowest number of MLGs (1) and the highest number (7) were found on trees with large, advanced SCN (Table 2).

As sampled trees 6 and 7 were the only trees with identical MLGs, the focus lies on these two sampled trees. In sampled tree 6, two SCN were identified, based on the externally visible top ends of the necrotic areas. For the majority of both SCN, L was isolated with the highest frequency. The other common MLGs, including M, N, O and P, were only found at the top end of the SCN (Fig. 5).

Sampled tree 7 (Fig. 6) showed the highest number of SCN as well as the highest number of MLGs. L, M, N, O and P MLGs were also present in sampled tree 7, while M and O were isolated with almost equal frequency. The distribution of MLGs in the stem collar of sampled trees 6 and 7 resembled a spatial clustering structure, because each SCN top end was dominated by a distinct MLG.

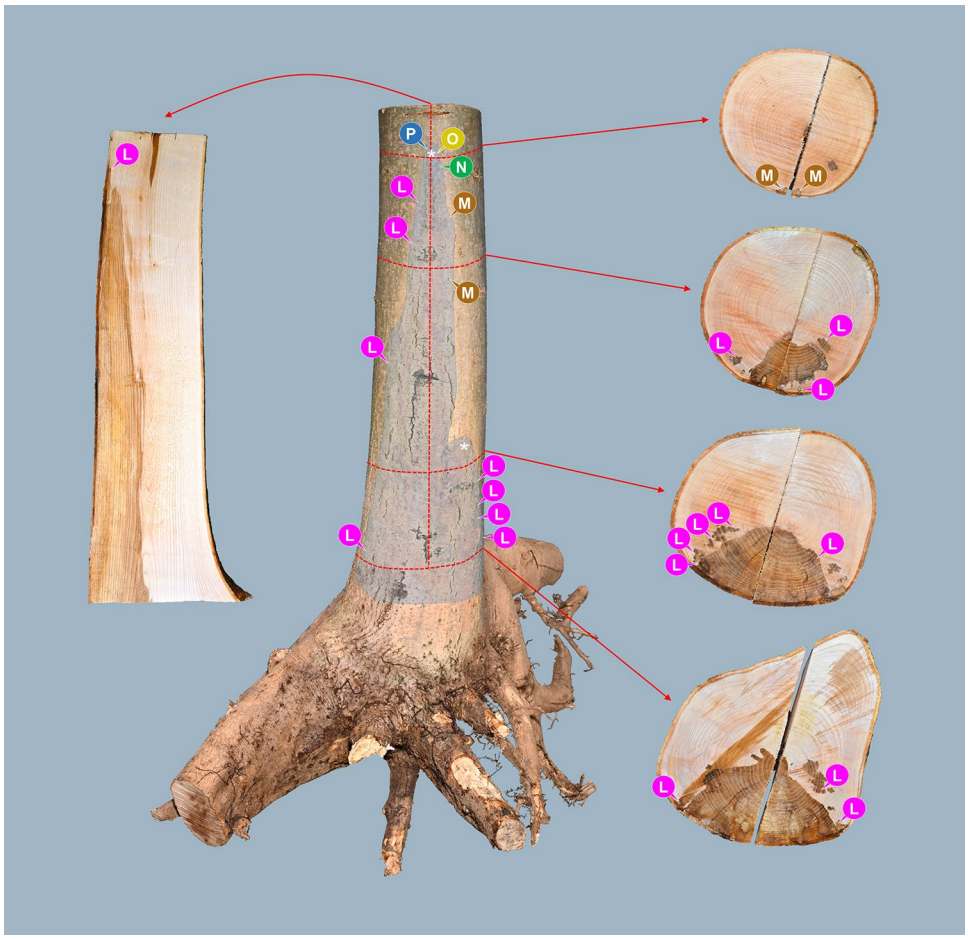

Fig. 5 Multilocus genotypes (MLGs) in sampled tree 6. Cuttings (dashed lines) and corresponding sections (arrow) are shown as red lines. The extent of the stem collar necroses from the outside is represented by the blue transparent area. The location of the different MLGs is indicated with corresponding colours and letters. Externally visible upper ends of necrotic areas are marked with white asterisks. In total, there were 27 *Hymenoscyphus fraxineus* isolation sites with five different MLGs

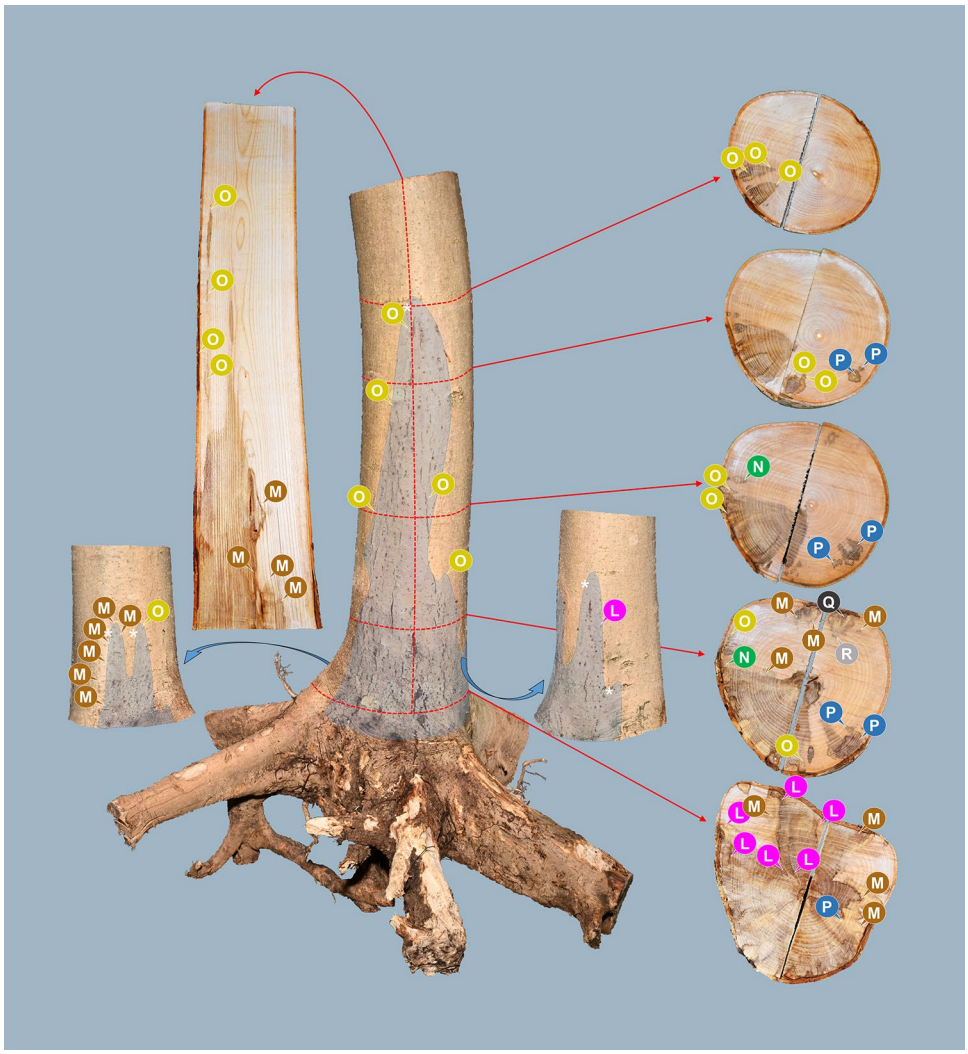

Fig. 6 Multilocus genotypes (MLGs) in sampled tree 7. Cuttings (dashed lines) and corresponding sections (arrow) are shown as red lines. The extent of the stem collar necroses from the outside is represented by the blue transparent area. The necrotic tissue at the back and the side of the stem are shown separately in their respective perspectives with blue arrow lines. The location of the different MLGs is indicated with corresponding colours and letters. Externally visible upper ends of necrotic areas are marked with white asterisks. In total, there were 55 *Hymenoscyphus fraxineus* isolation sites with seven different MLGs

Discussion

Identical multilocus genotypes in different sampled trees

One aim of this study was to determine the number of *H. fraxineus* individuals per SCN. In this context, it is important that the discriminatory power of the microsatellite assay used is high enough to distinguish individual MLGs. Resolution depends on the number of polymorphic loci analysed as well as on the number of alleles per locus. Most population genetic studies of *H. fraxineus* have used between 11 (Burokiene et al. 2015; Haňáčková et al. 2017) and 19 (Gross et al. 2012b; Meyn et al. 2019) microsatellite loci. While Meyn et al. (2019) were able to resolve all analysed samples, Gross et al. (2012b), Burokiene et al. (2015), and Haňáčková et al. (2017) reported identical MLGs. The trees analysed in Meyn et al. (2019) originated from a contiguous sampling site, where the maximum distance between analysed trees was 29 m. However, the total sample size of four trees was relatively small, decreasing the chance of finding identical MLGs.

There are several explanations to find identical MLGs. Asexual reproduction would lead to prevalence of a low number of genotypes at the population level. All available population genetics studies (e.g. Gross et al. 2012b, 2014b; Haňáčková et al. 2015, 2017; Burokiene et al. 2015) show a high genetic diversity at the population level supporting the current model of *H. fraxineus* reproducing exclusively sexually via ascospores (Gross et al. 2014a). In the present study, the presence of the same MLG in a stem collar necrosis of a single tree (Figs. 5, 6) is interpreted as mycelial growth originating from a single ascospore. However, it remains to be explained why the same MLGs are found on trees 6 and 7, because they cannot present a single contiguous mycelium. A possible explanation is the occurrence of twins during ascospore development. An ascus is dikaryotic, containing one nucleus from the male and one from the female reproductive structure. These two nuclei fuse via karyogamy to form a diploid nucleus. Subsequent meiosis produces four

haploid nuclei and is followed by mitosis, in which each nucleus divides again (Landvik et al. 2003). Thus, there are four times two identical spores in one ascus. Sampled trees 6 and 7 have a small distance between them (27 m) compared to the other pairwise tree distances (Fig. 4). Nevertheless, it is highly unlikely that two genetically identical spores would fall on these two trees.

The sampled trees with identical MLGs are located in sampling plot III with the highest density of ash trees and the most damaged crowns and stem collars (Online Resource 5). At this sampling plot, the pathogen population is the largest, which increases the probability of finding identical MLGs. *H. fraxineus* individuals from European populations exhibit predominantly biallelic loci (Gross et al. 2014b), implying a lower discriminatory power of the loci used, which increases the difficulty to distinguish between individuals. This is particularly severe when the individuals studied are closely related. The reanalysed data of Gross et al. (2012b) showed that in a full sibling population not all individuals can be fully resolved using a primer assay of 12 nuclear polymorphic markers. Therefore, the authors suggest that the identical MLGs in sampled trees 6 and 7 are closely related individuals and thus provide an indication of the answer to the question (ii) whether the same *H. fraxineus* MLGs in the SCN of different trees are different individuals. A higher genetic resolution could only be achieved with other methods, for instance, population genetics with whole genomes. A more traditional and morphology-based approach to determine whether the *H. fraxineus* individuals in sample trees 6 and 7 are very closely related or the same individual would be a mating test with mycelia of the different isolates in dual cultures. Such mating tests, not presented in this study, have been carried out, but due to the slow growth of *H. fraxineus* and the often highly variable culture habitus of a single strain, no reliable results could be obtained.

Colonisation patterns in stem collar necrosis

Another aim of this study was to determine whether the number of *H. fraxineus* individuals in a SCN would allow to differentiate between primary and secondary infections. The dominance of one MLG would be considered as a sign of primary infection causing the SCN, whereas an equal distribution of several MLGs would indicate secondary infection of an existing SCN. A tendency for one MLG to outnumber others within a tree and a spatial clustering structure of MLGs within a SCN were found in this study. This pattern is similar to the infection pattern found in ash shoots, where successful shoot infections are characterised by the dominance of one MLG (Haňáčková et al. 2017). Because *H. fraxineus* is the primary agent of ash dieback, which initially infects the shoot, the observed pattern is consistent with other studies suggesting *H. fraxineus* plays also a causal role in SCN emergence (Husson et al. 2012; Chandelier et al. 2016; Langer 2017). If *H. fraxineus* was a secondary coloniser, a more random distribution of MLG frequencies would be expected. Instead, the infection pattern probably established successively over time, assuming that all MLGs found in a SCN are successful pathogens, and the different MLGs probably represent multiple, temporally staggered infections. It is likely that the first MLG that successfully invades the stem become dominant because it has the temporal lead, which is then reflected in the size of the SCN.

Associated fungi and the cohabitation role of *Hymenoscyphus fraxineus*

As a continuation of the study by Peters et al. (2023), 23 additional MTs were found at the forest site Holzminden. Of these, three species, *Lopadostoma turgidum* (Pers.) Traverso, *Stachybotrys chlorohalonatus* B. Andersen & Thrane, and *Trimmatostroma betulinum* (Corda) S. Hughes, are described on ash for the first time. The detection of *L. turgidum* in woody tissues of ash is noteworthy, as Jaklitsch et al. (2014) could only find species of the genus *Lopadostoma* (Nitschke) Traverso in the bark of trees and shrubs, but never in underlying wood. The stromata of *L. turgidum* usually occur on *F. sylvatica*, which is one of the tree species present in the forest site Holzminden. (Chapter 2.1). *Stachybotrys chlorohalonatus* (*Stachybotryaceae*, *Hypocreales*) has been separated from *S. chartarum* (Ehrenb.) S. Hughes sensu lato as a distinct species, which differs morphologically and in the tri5, chs1 and tub1 gene fragment sequences (Andersen et al. 2003). This black mould was isolated from a cardboard on gypsum board in Denmark, a wet cellulose-containing material, the typical habitat of the species. *Trimmatostroma betulinum* (*Mollisiaceae*, *Helotiales*) is known to be a naturally occuring member of the fungal community that inhabits living leaves and twigs of *Betula* spp. without causing symptoms, but can also been found on living or dead twigs, branches and occasionally leaves of other plants including *Pinus*, *Alnus*, *Quercus,* and *Salix* species (UK, CAB International et al. 2003).

The 23 additional MTs represent approximately one-third of all fungi isolated (61) from the sampled SCN. This result confirms the hypothesis of Peters et al. (2023) that the number of associated species may increase as more different forest plots are examined. As expected, the most common fungi in this study (*Armillaria* sp., *Diaporthe* cf. *eres*, *D. fraxini*, *H. fraxineus*, and *Paracucurbitaria* sp.) match with the most common fungi isolated by Peters et al. (2023) and other previous studies on fungi associated with ash necroses (Lygis et al. 2005; Langer 2017; Linaldeddu et al. 2020). In contrast to the results of Langer (2017), Meyn et al. (2019), and Peters et al. (2023), the frequency and continuity of *Neonectria punicea* (J. C. Schmidt) Castl. & Rossman, represented by only two isolates, was significantly lower in this

study. However, similar to the results of Peters et al. (2023), at least a quarter of all MTs were identified as being able to decay wood (Online Resource 2).

The 274 *H. fraxineus* isolates and the occurrence and composition of the resulting 25 MLGs did not seem to be influenced by the presence of other isolated species or fungal diversity in any of the ten sampled trees. In general, a correlation would be conceivable, as *H. fraxineus* is a slow-growing fungus that could be overgrown by fast-growing fungi after their secondary colonisation (Kowalski and Holdenrieder 2009; Gross et al. 2014a). However, our results showed that neither the number of *H. fraxineus* isolates nor the number of MLGs were lower when the most common species, *Armillaria* spp. or *D. fraxini*, were present. The analysis showed that the fungal diversity (number of species) in SCN did not influence the composition of *H. fraxineus* or MLGs. This trend is unlikely to change, although theoretically more infections are possible, but individual strains may not be isolated from the wood.

The 61 MTs isolated included various endophytes, saprotrophs and pathogens. In this study, the most consistently detected isolates, besides *H. fraxineus*, belonged to the genus *Armillaria* (Fr.) Staude or could be identified as *D. fraxini*. It is likely that species of the genus *Armillaria* were present in European forests prior to ash dieback, as they are known to be widespread soil colonisers (Morrison 2004; Lygis et al. 2005; Bakys et al. 2009). Their common occurrence in advanced SCN can be explained by their ascribed property of being secondary pathogens that attack stressed trees and are able to decay wood (Chandelier et al. 2016). *Armillaria* spp. can colonise stem collars after SCN has been caused by *H. fraxineus*, just as *Armillaria* spp. can independently infect a weakened tree without SCN caused by *H. fraxineus* (Langer 2017). Irrespective of the individual infection process, this leads to an accelerated disease in ash trees affected by ash dieback. But even in the latter case, *Armillaria* spp. is only a secondary coloniser, because the tree has already been pre-damaged in the crown by the pathogen that causes ash dieback. *Diplodia fraxini*, on the other hand, is known to be an aggressive pathogen, causing bark lesions and wood discoloration or to enlarge necroses, which are primarily caused by *H. fraxineus* (Alves et al. 2014; Linaldeddu et al. 2020, 2022). Kowalski et al. (2017) ranked *D. fraxini* as the second most pathogenic fungus after *H. fraxineus*, although it was not mentioned as a frequent coloniser of *F. excelsior* prior to ash dieback (Kowalski et al. 2016). *Diplodia fraxini* undoubtedly contributes to a greater extent of damaged stem collars and therefore plays an important role in the development of this disease.

Other, less common fungi, such as *Neonectria punicea*, can occur as latent pathogens, meaning that they are already present in the tree as endophytes prior to damage and change their life cycle to become pathogenic when the host is weakened (Langer 2017). Such fungal characteristics may also increase SCN development by weakening the tree in the context of ash dieback.

Conclusion and outlook

The study shows that in most sampled trees one MLG outnumbers the others, indicating that a SCN was initiated by a single *H. fraxineus* MLG. It is therefore suggested that *H. fraxineus* is a primary coloniser of SCN. The following conclusions can be drawn:

1. Because of the close proximity, it is reasonable to assume that identical MLGs in a SCN result from growth of a single individual rather than multiple infections.
2. Identical MLGs in different trees could result from infections of closely related individuals.
3. Multiple MLGs within one tree may represent multiple successive infections over time.

In this study, the authors have shown the limitations of using microsatellites to resolve individuals. In future population genetic studies, other molecular markers that cover larger genome sections should be used, especially when close genetic relationships are suspected. One method could be single-nucleotide polymorphism analysis based on genome sequencing (population genomics).

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest. There are only non-financial research interests, related directly or indirectly to the work submitted for publication here.

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4. General discussion

Ash dieback has been a growing threat to trees of common ash in Germany for many years. The economic and security implications, as well as the impact on the biodiversity of our forest ecosystems, are immense. Not least because stem collar necroses contribute significantly to the problem. The reduced stability caused by basal damage leads not only to massive dieback and falling trees, but also to economic losses, a decline in the organisms associated with healthy ash trees, and a significantly increased risk in stands containing ash trees.

4.1. Fungi associated with stem collar necroses

The fungi associated with stem collar necroses are very diverse and it is likely that new species will be identified as more ash stands are sampled. However, there are only a few fungal species that are consistently isolated with high frequency from stem collar necroses and are therefore of particular importance (Lygis et al. 2005; Enderle et al. 2017; Langer 2017; Meyn et al. 2019; Peters et al. 2023). The current extent of ash dieback, including its damage and aftereffects, was only made possible by the combination of the primary pathogen, *H. fraxineus*, with the high incidence of secondary fungal species. For example, the acceleration of necrosis by *Armillaria* species or *D. fraxini* - without these commonly associated (opportunistic) pathogens, the extent of disease severity would probably be significantly lower than with them. Even if the interactions between the different fungi and possible influences of abiotic factors on the occurrence of individual species are not yet fully understood, a characterisation of the ecological functions of the most common fungal species is already possible based on the current state of research (Kowalski et al. 2017; Kubiak et al. 2017; Karadžić et al. 2020).

The results of this work made an important contribution to the previously largely unknown fungal biodiversity of ash wood in Germany. A number of fungal species have been described for the first time on common ash, including *Cryptostroma corticale* (Ellis & Everh.) P.H. Greg. & S. Waller, the causal agent of sooty bark disease of maple, and *Diplodia sapinea* (Fr.) P. Karst., the causal agent of pine shoot dieback and *Diplodia* shoot dieback of conifers (Peters et al. 2023). Furthermore, a new ascomycete species was discovered and described for the first time: *Vexillomyces fraxinicola* S. Bien, S. Peters & G. Langer (Tan et al. 2022).

4.2. The role of *Hymenoscyphus fraxineus* in stem collar necrosis

Most stem collar necroses on common ash are primarily caused by the ash dieback pathogen *H. fraxineus*. Next to the associated secondary fungi, it therefore plays the most important role in the damage caused by the ash dieback disease. It initiates the dieback process in the crown and at the base of the stem, thus creating the conditions for the colonisation of other fungi. There is therefore no doubt about its key role. As well as interacting with secondary fungi, the presence of different *H. fraxineus* strains in stem collar necroses must be considered. Understanding this structure is necessary to understand the fungus, including its infection biology and distribution mechanisms, and to use this knowledge to potentially develop appropriate control measures. The ability of different strains of the *H. fraxineus* pathogen to invade both the shoots and the stem base has been demonstrated in several studies (Haňáčková et al. 2017b; Bengtsson et al. 2014; Meyn et al. 2019). It is assumed that one strain of *H. fraxineus* causes the damage and then further infections by other strains of the fungus successfully invade at different times. This is supported by the study of Peters et al. (2024) where one genotype per stem collar necrosis dominated. The extent of damage is likely to depend more on the aggressiveness of individual strains (Kosawang et al. 2020) and possibly intraspecific competition than on the number of different genotypes.

4.3. Conclusion and outlook

The results of this study provide a better understanding of the cause and development of stem collar necroses on ash. Insight into the aetiology of stem necroses by determining the community of associated fungi and their characterisation in terms of their ecological functions and dependence on site conditions provides important knowledge for ash dieback research. In general, the fungal biodiversity in the woody tissues of ash trees in Germany has become more comprehensible due to the extensive studies.

Building on the results, further studies of this kind should be carried out, for example to supplement and validate the range of sites. Furthermore, investigation of new forest sites is likely to increase knowledge of ash wood biodiversity by identifying additional species involved. Fungal species about which little is known so far can be studied in more detail for the ecological function they perform (pathogenicity tests, wood rot tests). This would allow a better assessment of the risk of infestation in terms of affecting stability and resistance to breakage,

or traffic and work safety of affected ash trees, and would therefore be of high practical relevance.

In addition, the isolated and live archived fungi represent a genetic resource and a potential source of new natural products or antagonists. The methods that have been proven to be suitable for the isolation and characterisation of wood-associated fungi can be used in the future as a standard method for the analysis of emerging fungal or complex forest tree diseases.

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Anlage

Erklärung über den Eigenanteil an den Schriften

Manuskript I

Probenahme	Sandra Peters, Sebastian Fuchs
Laborbearbeitung	Sandra Peters, Steffen Bien
Analysen	Sandra Peters, Sebastian Fuchs
Manuskripterstellung	
Erster Entwurf	Sandra Peters
Ergänzende Überarbeitungen	Steffen Bien, Johanna Bußkamp, Gitta Jutta Langer, Ewald Johannes Langer

Manuskript II

Probenahme	Sandra Peters
Laborbearbeitung	Sandra Peters, Steffen Bien, Nina Gruschwitz, Viktoria Blunk
Analysen	Sandra Peters, Nina Gruschwitz
Manuskripterstellung	
Erster Entwurf	Sandra Peters
Ergänzende Überarbeitungen	Nina Gruschwitz, Steffen Bien, Sebastian Fuchs, Ben Bubner, Gitta Jutta Langer, Ewald Johannes Langer

Die Angaben müssen von den Mitautoren schriftlich bestätigt werden:

Ich bestätige die von Frau Peters zum Eigenanteil abgegebene Erklärung:

Name: Unterschrift:

Sebastian Fuchs

Steffen Bien

Johanna Bußkamp

Gitta Jutta Langer

Ewald Johannes Langer

Nina Gruschwitz

Ben Bubner

Viktoria Blunk

Zusätzliche Veröffentlichungen

im Zusammenhang mit dem Forschungsprojekt dieser Dissertation in chronologischer Reihenfolge

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Erklärung gemäß § 8 der Allgemeinen Bestimmungen für Promotionen der Universität Kassel vom 14.07.2021

1. Bei der eingereichten Dissertation zu dem Thema „Ash dieback – The pathogen *Hymenoscyphus fraxineus* and other fungi associated with stem collar necroses of common ash“ handelt es sich um meine eigenständig erbrachte Leistung.
2. Anderer als der von mir angegebenen Quellen und Hilfsmittel habe ich mich nicht bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen veröffentlichten oder unveröffentlichten Werken übernommene Inhalte als solche kenntlich gemacht.
3. Die Dissertation oder Teile davon habe ich

   ☒ bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.

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   Titel der Arbeit:
   Hochschule und Jahr:
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   Es handelt sich dabei um folgenden Teil der Dissertation:

4. Die abgegebenen digitalen Versionen stimmen mit den abgegebenen schriftlichen Versionen überein.
5. Ich habe mich keiner unzulässigen Hilfe Dritter bedient und insbesondere die Hilfe einer kommerziellen Promotionsberatung nicht in Anspruch genommen.
6. Im Fall einer kumulativen Dissertation: Die Mitwirkung von Koautoren habe ich durch eine von diesen unterschriebene Erklärung dokumentiert. Eine Übersicht, in der die einzelnen Beiträge nach Ko-Autoren und deren Anteil aufgeführt sind, füge ich anbei.
7. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.

13.03.2024 _____

Datum Unterschrift