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Running title: Sampling threatened deadwood fungi

How to best detect threatened deadwood fungi – comparing metabarcoding and fruit body surveys

Abstract

Effective conservation strategies are needed to prevent further loss of biodiversity. This requires a comprehensive assessment of species' presence, distribution, and population sizes. Such assessments can be extremely challenging for species-rich taxa, like fungi, which are difficult to detect and identify. In recent years, metabarcoding applied to environmental samples has proven to be a promising method for fungal detection. However, its potential against traditional fruit body surveys in monitoring threatened fungal species has rarely been tested. Here, we utilized data on deadwood-inhabiting fungi from 569 deadwood objects across five sites in Central Europe to compare the effectiveness of fruit body surveys and metabarcoding (of low and high sampling intensity) in detecting threatened species. Across objects, sites, and per object, metabarcoding was more effective in detecting threatened species than fruit body surveys, regardless of sampling intensity. Eleven percent of all threatened species across all objects and sites could be detected by both methods, 70% exclusively by metabarcoding and 18% exclusively by fruit body surveys. The number of species detected by both methods on the same object was <4%. Effects of high sampling intensity metabarcoding were stronger than low sampling intensity at object level. The effectiveness of the survey method was mainly independent of deadwood object characteristics. We suggest that metabarcoding is a valuable tool for threatened fungal species monitoring and conservation status assessments. However, for a comprehensive assessment, fruit body surveys are still needed, as this method detected a unique set of species and indicates the presence of vital fungal individuals.

Keywords: species conservation, deadwood-inhabiting fungi, fungal conservation, species detection, Red List

Highlights:

- Metabarcoding is an efficient method to detect threatened fungi in deadwood
- Metabarcoding and fruit body surveys detect different threatened fungal species
- To monitor threatened fungi, metabarcoding and fruit body surveys should be combined

1 Introduction

Human impact on Earth's ecosystems is causing a biodiversity crisis (Nic Lughadha et al., 2020). Despite various conservation efforts, global biodiversity continues to decrease (Cowie et al., 2022). This underpins the need for monitoring biodiversity at relevant spatiotemporal scales to evaluate the presence, distribution, and population dynamics of threatened species. However, research mainly focused on well-known species groups (Lindenmayer and Likens, 2011; Troudet et al., 2017), while taxa that are difficult to detect or identify remain understudied. Hence, for improved biodiversity conservation of such taxa, we need rapid and cost-efficient sampling methods providing data with high spatial and temporal resolution (Hochkirch et al., 2021).

One of these hyperdiverse and largely understudied taxonomic groups of interest is fungi. With ca. 140,000 described species, this group is currently the third-richest eukaryotic kingdom, with a predicted species richness of over 6 million (Baldrian et al., 2022). Moreover, fungi are among the most important organisms driving global primary production and the carbon- and nutrient cycle (Boddy and Watkinson, 1995). Still, conservation frameworks largely neglect fungi mainly because of the limited knowledge of their diversity and distribution (Heilmann-Clausen and Vesterholt, 2008; Hochkirch et al., 2021).

Surveying fungal fruit bodies is the traditional monitoring technique used over decades, but has its shortcomings. Fruit bodies of some fungal species are highly ephemeral, as their production depends on intrinsic factors and specific environmental conditions (e.g., weather, Sakamoto, 2018). Therefore, different species can have specific intra-annual (Halme and Kotiaho, 2012; Purhonen et al., 2017) and irregular inter-annual phenologies (van der Linde et al., 2012; Moore et al., 2008). In addition, some species produce small and inconspicuous fruit bodies that are easily overlooked even when present (Lõhmus, 2009), and others need years to collect enough resources for fruit body production (Ovaskainen et al., 2013). Finally, the determination of fungal species can be laborious and complicated (e.g., deep exploration of macro- and microscopic characteristics, availability of specific determination keys) and thus can be prone to identification errors. Note that for many species and

specific fungal groups (e.g., corticioid fungi), long-term experience is necessary for a reliable identification of species.

An alternative, more recently emerged approach to fruit body surveys is species identification using metabarcoding techniques applied to environmental samples (eDNA). The major advantage of metabarcoding is that it allows the detection of fungal species without visible fruit bodies (Rajala et al., 2011; Kubartová et al., 2012; Fischer et al., 2012). Further, expert knowledge to identify fungal specimens is not necessary and sampling can be easily standardized. However, it requires detailed understanding of the limitations of metabarcoding and bioinformatics (Thomsen and Willerslev, 2015). The limitations include the possibility of technical or biological noise, e.g., low-quality or chimeric sequences (Lindahl et al., 2013), remnant DNA from dead organisms (Rajala et al., 2011), or randomly present nonviable species (Tuovinen et al., 2015). More generally, reliable species identification from metabarcoding depends on the used lab- (e.g., primers, markers) and bioinformatic procedures (e.g., clustering method, bioinformatic pipelines, reference database). Finally, only a minimal amount of substrate is usually sampled and some species may remain undetected due to high spatial heterogeneity in the fungal abundance and due to physiological properties of fungal species (e.g., those with small mycelia; Kubartová et al., 2012; Ovaskainen et al., 2010). Thus, the effectiveness of metabarcoding might depend on the sampling intensity. In addition, species identification via metabarcoding depends on the completeness and quality of the reference databases (Hibbett et al., 2016).

As a possible solution for improving conservation assessments, a combined use of fruit body surveys and metabarcoding methods has been discussed for a decade (Runnel et al., 2015). However, empirical tests on how to do this effectively are scarce. In the case of soil fungi, metabarcoding has shown higher efficiency in detecting threatened species, given their presence in DNA reference databases; whereas fruit body surveys had the advantage of also detecting species lacking reference sequences (Frøslev et al., 2019). To date, comparative studies from other important fungal habitats, such as deadwood, are lacking (but see Ovaskainen et al., 2013, Saine et al., 2020). Deadwood-

inhabiting fungi are among the most threatened organisms in forests due to centuries of deadwood removal and timber extraction (Siitonen, 2001) and their species numbers continue to decline (Nordén et al., 2013, Tikkanen et al., 2006), while fungal conservation only recently received broader attention (Mueller et al., 2022). Efficient monitoring concepts might help prevent deadwood-inhabiting fungal species from further decline via a rapid evaluation and adaptation of conservation strategies.

Our study aimed to compare the efficiency of fungal fruit body surveys and metabarcoding in detecting threatened fungal species in deadwood. The comparison will provide insights into the advantages and limitations of both methods in species assessment, leading to improved conservation management of forest species. We used an extensive dataset comparing records of threatened fungi based on fruit body surveys and metabarcoding of two different sampling intensities (different number of drilling cores per object) from 569 deadwood objects in five forest sites in Central Europe (Fig. 1) sampled over several years. We expect that the efficiency of metabarcoding in detecting fungal species depends on the presence of DNA references in the databases (Frøslev et al., 2019). Reference sequences including rare species might have increased in recent years due to molecular advancements (Lewin et al., 2018). Further, metabarcoding allows detecting species based on their mycelium which is less ephemeral than fruit bodies. We, therefore, overall hypothesize that metabarcoding outperforms fruit body surveys in detecting threatened fungal species in deadwood. Specifically, we asked: (i) Which method detects threatened species per site/deadwood object more efficiently? (ii) How often do both methods detect the same species simultaneously on the same object? (iii) Which method better detects threatened species regarding specific deadwood characteristics (decay stages, angiosperm/gymnosperm)? (iv) Which method detects threatened species on more objects? In all study questions, we further aim to assess whether the sampling intensity of metabarcoding impacts the results. Our study defines threatened species as “near threatened” or higher ranks in Central European Red Lists (Dämmrich et al., 2016; Holec and Antonín, 2006).

2 Materials and Methods

2.1 Study areas and sampling

We used fruit body and metabarcoding data from five different sites in Central Europe (Fig. 1). These sites represent different environmental conditions from old-growth to managed forests and deadwood from different tree species and different decay stages: The study sites Swabian Alb (Alb), Hainich-Dün (Hainich), and Schorfheide-Chorin (Schorfheide) are located across Germany and are part of the BELongDead experiment (Kahl et al., 2017) set up in the Biodiversity Exploratories (Fischer et al., 2010). The deadwood objects consisted of four conifers (*Larix decidua*, *Picea abies*, *Pinus sylvestris*, and *Pseudotsuga menziesii*) and nine deciduous (*Acer* spp., *Betula pendula*, *Carpinus betulus*, *Fagus sylvatica*, *Fraxinus excelsior*, *Populus* spp., *Prunus avium*, *Quercus* spp., and *Tilia* spp.) tree genera. Note that due to the manipulation of hundreds of coarse woody debris objects across a large area within Germany, some uncertainties occurred for a few tree genera that not the exact same tree species had been exposed (e.g., *Quercus robur* vs. *Quercus petraea*). As this is rare in our dataset, particularly since it applies only to a few species, and the fact that we compared the sampling methods at each object simultaneously, we expect that this is not relevant to the validity of the comparison of methods. Of each tree species, respectively genera, nine replicates (twelve in Hainich) were placed within each site. The study site Bavarian Forest is located within the Bavarian Forest National Park in south-eastern Germany, here data from 34 deadwood objects of *Fagus sylvatica* and 33 objects of *Abies alba* from a larger deadwood experiment was used (Krah et al., 2018). The study site Žofín is located in the Novohradske Hory mountains (Czech Republic), in the core zone of the Žofínský prales National Nature Reserve, a natural forest with no human intervention since 1838. At this site, no standardized deadwood was placed, but 34 *F. sylvatica*, 43 *Picea abies*, and 35 *A. alba* deadwood objects from trees that have died naturally were sampled (Baldrian et al., 2016). For further details on the study sites, see Table 1.

2.1.1 Fruit body surveys

Fungal fruit bodies were recorded during the peak fruiting season (September-October, see 2.3 for sensitivity analysis) in all study sites. In Alb, Hainich, and Schorfheide sampling took place in 2012, 2015, and 2018, in the Bavarian Forest in 2012, 2013, and 2015, and in Žofín in September 2017. Fungi were determined directly in the field or using microscopy in the laboratory (e.g., in the case of small Ascomycetes or corticioid species). In all sites, fruit body surveys were carried out by the same team of experienced field mycologists (see Acknowledgements).

2.1.2 Wood sampling for metabarcoding and sample processing

The wood samples for metabarcoding were in most cases collected within the same year as fruit bodies, but in the Alb, Hainich, and Schorfheide the last wood samples were collected one year earlier (in 2017, see 2.3 for sensitivity analysis). To consider sampling intensity (number of drilling cores per object) in the analysis, sites were grouped into low-sampling intensity (one drilling core: Alb, Hainich, and Schorfheide) and high-sampling intensity (four drilling cores: Bavarian Forest and Žofín). In Alb, Hainich, and Schorfheide, a wood sample per object was collected at one position in each sampling campaign. An auger (20 mm) was inserted into the center of each object with an angle of ~45° to the vertical plane. In the Bavarian Forest and Žofín, a vertical sample across the entire diameter was taken at 1/8, 3/8 5/8, and 7/8 of the object length with an auger (8 mm); in the Bavarian Forest all drilling cores were pooled in one bag, in Žofín two adjacent cores were collected in one bag each. In all sites, if present, the outer bark was removed and the object surface was cleaned with ethanol before drilling and the auger was sterilized between drillings by rinsing with ethanol and burning. Samples were frozen within a few hours after drilling at -40 °C. See references in Table 1 for detailed information about sample processing (DNA extraction, PCR, and sequencing) and 2.3 for sensitivity analysis concerning different sampling strategies.

For this study, amplicon sequencing data was unified for all samples and they were processed using the pipeline Seed 2.1.2 (Větrovský et al., 2018). Briefly, paired-end reads were joined using fastq-join (Aronesty, 2013). The ITS2 region was extracted using ITSx 1.0.8 (Nilsson et al., 2010) before

clustering and chimera detection. Chimeric sequences were detected and deleted using USEARCH 64-bit v.11.00.667_i86linux64 (Edgar, 2010), and sequences were clustered into operational taxonomic units (OTUs) using UPARSE implemented in USEARCH (Edgar, 2013) at a similarity level of 97%. The most abundant sequence of each OTU was selected as a representative. Taxonomic identification was performed using BLASTn against UNITE 9.0 (Nilsson et al., 2019). The best identification was selected based on the highest bitscore, lowest e-value, and highest similarity. This reduces sequences with highly gapped alignments and, thus, uncertainties in species identification. To ensure that the species assignment is most probably correct, only OTUs with >99% sequence similarity were considered in the further analyses. However, results can be sensitive against the used threshold of similarity and alignment coverage and whether singletons are considered or not (see 2.3 for sensitivity analysis).

2.2 Data preparation

To compare the sites, we combined the Red Lists of threatened Fungi in Germany (Dämmrich et al., 2016) and the Czech Republic (Holec and Antonín, 2006) to create a regional Red List, and extracted threatened species in this list from both the fruit body and metabarcoding datasets. We used MycoBank (Crous et al., 2004, version from 21st March 2023) to unify the nomenclature between the databases.

We aggregated the OTUs that represented the same threatened species. The further analyses were based on the presence-absence of such species per deadwood object at each sampling event. Species functional groups were assigned with FungalTraits (Põlme et al., 2020). A complete species list with the number of objects the species were detected on is available in Table A1. As suggested by Frøslev et al. (2019), we excluded 19 species from the fruit body dataset which had no reference sequences in UNITE 9.0, as these species could not be detected by metabarcoding. This standardization assumes that all species are potentially detectable using both methods. Thus, we avoid that our results might be

temporarily vague and not be valid anymore in near future with progressing reference sequences. However, as 19 missing reference sequences are a considerable amount that should not be ignored in a comparative method study, we provided additional analyses considering these species (see section 2.3).

2.3 Data Analyses

All analyses were conducted using R 4.2.2 (R Core Team, 2022). All Euler plots, except Fig. 5, were created using the package *eulerr* (Larsson, 2021). Fig. 5 was created with the package *ggplot2* (Wickham, 2016).

To explore the total number of threatened species detected by each method, we counted the number of threatened species sampled by each method within and over all sites of the same sampling intensity and calculated the share. To identify differences in the efficiency of each method on object level, we used generalized linear mixed-effects models for negative-binomial distributed count data (function *glmer.nb*, package *lme4*, Bates et al., 2015). We used the number of threatened species detected per occupied object per sampling campaign as a response and sampling method as an explanatory variable; "object ID" was used as a random factor. We used coarse woody debris in our study with roughly similar sizes. However, as the detection probability of species can depend on the size of an object, we considered deadwood volume (m^3) as a covariate in our models.

To investigate whether both methods detect the same threatened species simultaneously on the same object, we calculated the number of simultaneous detections per object and sampling year and set it in relation to the total number of detections.

To explore whether the number of detected threatened species per method depended on deadwood characteristics (decay stages 1 to 4 (Bässler et al., 2010), angiosperm/gymnosperm), we calculated the overall number of threatened species detected by each method and the share for each characteristic over all sites of the same sampling intensity. Further, we tested if the number of threatened species

sampled by either method per study object depended on their decay stages or identity (angiosperm or gymnosperm). This was again done using generalized linear mixed-effects models for negative-binomial count data (*glmer.nb*), where the method was included as an explanatory variable and "object ID" was the random effect.

Finally, to investigate if one of the methods is more efficient in detecting occupied objects, we calculated the number of occupied objects detected by each method, within and over all sites of similar sampling intensity. Additionally, on object level, we utilized generalized linear mixed effects models with the function g*lmer* and a binomial distribution in the package *lme4* (Bates et al., 2015). As response variable, we coded if a method detected an object occupied by at least one threatened species with 1 (detected) or 0 (not detected). The sampling method was used as the explanatory variable, and "object ID" as a random effect.

We are aware that our data set has some limitations. Therefore, we applied the following sensitivity analysis: (i) We applied one fruit body survey during the main fruiting season in all sites (see 2.2.1). However, in the Bavarian Forest, we have additional data from a second survey in Spring (April-May) for 2012, 2013, and 2015. As it has been recommended to repeat fruit body surveys per log and season to increase sampling efficiency (Abrego et al., 2016), we compared the performance of the methods based on data from the main fruiting season versus the data additionally considering the spring survey from the Bavarian Forest site. This exercise revealed that our overall inferences based on the data using one main fruiting peak in the year were robust (Table B1). (ii) In the sites Alb, Hainich, and Schorfheide sampling years do not perfectly match and data from 2017 is only available for metabarcoding and data from 2018 only for fruit bodies. To fully standardize our data, we excluded data from these years and re-run analyses. As this exercise revealed similar results (Table B2), we present the results of the full dataset. (iii) Results from metabarcoding can be sensitive against the used threshold of coverage and similarity and whether singletons are considered or not. Therefore, we applied analysis based on 97% and 99% similarity thresholds for the data sets using all OTUs and the data set in which we excluded global singletons. Furthermore, we applied a statistical singleton

correction procedure of potential sequencing errors based on the distribution of OTUs and reads of each sample (Table B3) as suggested by Chiu and Chao (Chiu and Chao, 2016). Lastly, we conducted our main analyses with data using a ≥ 90% alignment threshold to the metabarcoding data, this excluded sequences with high identity in a very small region and is a more conservative species annotation approach (Table B4). Overall, results were consistent when accounting for different similarity (percent identity) levels, whether global singletons were excluded or not, or when using a ≥ 90% alignment threshold. Therefore, for the final analyses, we used the complete data set, without excluding singletons or accounting for alignment coverage and applying the >99% similarity threshold. (iv) For reasons of standardization, we excluded 19 species that have no reference sequence (Frøslev et al., 2019, see 2.2). However, as this exclusion causes bias towards the metabarcoding approach (i.e., proportion of species detected by fruit body surveys might be underestimated), we ran our analysis based on all threatened species and revealed overall consistent results (Table B5). (v) The overall aim of the analyses was to compare the efficiency of the two study methods in detecting threatened species across and within sites and objects. However, we acknowledge that the sites considered represent specific study settings and that first, environmental conditions, log characteristics, and second, sampling strategy are confounded (see 2.1). To address the first issue, we considered each site separately in the main analyses and compared the effects with the overall effects. The second issue relates to the fact that we were interested in separately testing metabarcoding data for low (one drilling hole per study object; sites: Alb, Hainich, and Schorfheide) versus high sampling intensity (four drilling holes per object; sites: Bavarian Forest, Žofín), as this is a simple approach easily applicable in the field and hence, potentially interesting for conservation. Note, however, that in the Bavarian Forest, we pooled all four drillings with further processing of one composite sample. In Žofín, we pooled the four drillings into two composite samples (each based on adjacent drilling) with further separate processing (see 2.1). Therefore, sampling intensity in our study is based on the number of drillings per object. Note that the different procedures in the Bavarian Forest and Žofín did not affect our results. We found overall consistent patterns when comparing the results with our

overall approach, i.e., contrasting one versus four drillings (Fig. 2, Fig. A1). (vi) Finally, as the data for the different sites were from different years and our analyses were based on aggregated data per site, we provide the overall results for each year separately. This exercise demonstrates variability in the performance of the methods, however, yielded overall consistent results (Table B6).

3 Results

In total 166 threatened fungal species were detected; fruit body surveys detected a total of 64 species and metabarcoding detected 124 species (note that species' numbers per method are not exclusively, thus, the same species can add to the count in both methods). Nineteen fungal species missed reference sequences and had to be excluded for standardization (see. 2.3). Thus only 45 fungal fruiting species were considered in the following analyses. Nevertheless, we found consistent results, when accounting for these species (Table B5). In the Alb 35 threatened fungal species were detected (fruit body survey: 10/metabarcoding: 27). In Hainich 36 (11/27), in Schorfheide 32 (7/28), in the Bavarian Forest 66 (3/64), and in Žofín 64 (30/47) threatened fungal species were detected, respectively.

3.1 Which method detects threatened species per site/deadwood object more efficiently?

Overall, more threatened species were detected by metabarcoding than by collecting fruit bodies, regardless of the intensity of sampling. (Fig. 2a-b). The number of shared species between fruit body surveys and low-sampling intensity metabarcoding was low (7.9%) and only slightly higher for fruit body surveys compared with high-intensity metabarcoding (14.7%). The proportion of species detected by both methods varied between 1.5% and 20.3% among the sites regardless of metabarcoding sampling intensity (Fig. 2 b-g). Each method sampled a unique set of species (Table A2-A6). Metabarcoding of both sampling intensities also detected more threatened species on each deadwood object than fruit body surveys (Table 2).

3.2 How often do both methods detect the same species simultaneously on the same object?

The different sampling methods rarely detected the same species simultaneously on the same deadwood object (Fig. 3). Only one species, *Agaricus laevigatus* (syn. *Mycena laevigata*), was detected frequently (19 times) simultaneously by both methods in the site Žofín (Table A6).

3.3 Which method better detects threatened species regarding specific deadwood characteristics (decay stages, angiosperm/gymnosperm)?

Metabarcoding of both sampling intensities detected a higher number of threatened species than fruit body surveys independent of the decay stage (Fig. 4). The proportion of those species detected uniquely by either method or shared differed among decay stages. Compared to metabarcoding of low sampling intensity, fruit body surveys were most efficient in decay stage one (Fig. 4a, c, e, g). In turn, in the case of high-intensity metabarcoding, fruit body surveys detected most species in decay stages two to four (Fig. 4b, d, f, h).

Across all sites, metabarcoding also detected more threatened species on both gymnosperms and angiosperms than fruit body surveys (Fig. 5). Metabarcoding of low sampling intensity uniquely detected around 57.2% of all species in angiosperms and 42.9% in gymnosperms. Metabarcoding of high sampling intensity uniquely detected 55.5% of all species in angiosperms and 59.2% in gymnosperms. Fruit body surveys detected 17.5% of all species in angiosperms uniquely when compared with low sampling-intensity metabarcoding and 9.6% when compared with high-intensity metabarcoding. In gymnosperms, fruit body surveys detected 7.5% and 9.3%, respectively.

On the object level, metabarcoding of high sampling intensity detected more threatened species than fruit body surveys independent of the decay stage and whether the host tree was angio- or gymnosperm (Table 3). However, metabarcoding of low sampling intensity did not detect significantly more threatened species than fruit body surveys in decay stages two and three.

3.4 Which method detects threatened species on more objects?

Overall, per site and on object level metabarcoding detected threatened species on more objects than fruit body surveys (Table 2, Fig. 6, Fig. A1). Fruit body surveys detected uniquely more species on objects when compared with low-intensity metabarcoding than when compared with high-intensity metabarcoding. The number of objects both methods detected threatened species on was 17.2% of all occupied objects when fruit body surveys were paired with metabarcoding of low sampling intensity, and 33.9% when compared with high-sampling intensity metabarcoding.

4 Discussion

Deadwood is a critical habitat of central importance for species conservation (Siitonen, 2001, Stokland et al., 2012). To provide guidance for efficient monitoring of threatened fungal species in nature conservation, we compared the sampling methods of fruit body surveys and metabarcoding. Across and within objects, we found metabarcoding outperforming fruit body surveys. However, approximately 3-26% of the threatened species detected per site were only surveyed as fruit bodies. This range increased to 6.5-38.6% when considering 19 species found via fruit body surveys but lacking reference sequences (Table B5). Further, more detailed analyses revealed that the number of simultaneous species detection by both methods on the same object was very small. Hence, both methods provide complementary information for a more comprehensive picture of the occurrence of threatened fungal species.

4.1 Efficiency of metabarcoding in detecting threatened species

Metabarcoding detected more threatened fungal species than fruit body surveys independent of sampling intensity. This is in line with a recent study showing that, when only species present in eDNA reference databases were considered, metabarcoding was slightly more efficient than fruit body surveys in detecting threatened soil fungi (Frøslev et al., 2019). The simplest explanation could be that fungi are always present as mycelium but only temporarily visible and detectable as fruit bodies (e.g., Frøslev et al., 2019; Ovaskainen et al., 2013; Saine et al., 2020), which increases the probability of

detection via metabarcoding. However, to overcome this limitation, Abrego et al. (2016) recommended repeating fruit body surveys over time if the aim is to record the total species richness in a deadwood object or to estimate the population size of a species. In our study, we carried out one fruit body survey per year in the main fruiting season for two to three years. We, thus, potentially missed species that fruited outside the main fruiting season. Nevertheless, when including data from an additional spring sampling campaign in the Bavarian Forest, which we formerly excluded due to standardization, fruit body surveys did not detect more species (Table B1). The occupancy of already detected species increased slightly, but this did not alter the main findings. We, therefore, cautionary conclude that the sampling intensity of fruit bodies in our study might not explain the lower performance in comparison to metabarcoding. However, increasing the number of fruit body surveys per object continuously within a season at a temporal finer scale across years may improve the efficiency of fruit body surveys detecting threatened species (Abrego et al., 2016).

Another explanation for the better performance of metabarcoding might be that 11% of all detected threatened fungal species (19 species) had no database reference sequence – thus were undetectable with this method. We excluded these species prior to the main analyses for reasons of standardization (see 2.3 Data Analyses and Frøslev et al., 2019). Ignoring these species might lead to an overestimation of the detection probability by metabarcoding relative to fruit body surveys. However, when considering these species in our main analysis, metabarcoding still outperforms fruit body surveys in detection efficiency (Table B5). The missing species in our study further highlight the importance of collecting reference sequences to complete databases, as apparently even threatened species in supposedly well-covered areas are missing (Abarenkov et al., 2018).

Even though we showed that metabarcoding outperforms fruit body surveys, species identification based on metabarcoding and database matching might be false positive/negative due to methodological limitations. There are several possibilities for how errors can arise: (i) Sequences can be subject to sequencing errors and thus deviate from biological reality. However, while errors may arise during high-throughput sequencing (HTS), they are quite rare with paired-end Illumina

sequencing. We further expect most sequences to be biological correct, as the most abundant sequence within each molecular taxon represents a large proportion of all sequences (e.g., >50%, in this dataset) and was used for taxonomic annotations. Since errors in HTS occur randomly, we assume that it is less probable that a frequently observed sequence is erroneous. Moreover, it is rather improbable that errors in sequencing change the sequence in a way it resembles a sequence of another existing taxon (false negative; Větrovský et al., 2016). Hence, we assume that potential sequencing errors might not change the main inferences of our study. (ii) Closely related species can be indistinguishable using the molecular marker applied (i.e., two or more species have an identical or a highly similar sequence within the used molecular marker). However, the genetic distance between fungal species based on the ITS region is typically relatively large, making species identification possible (Schoch et al., 2012). Yet, we cannot fully exclude false positive and negative assignments. Further, the performance of genetic markers differs among fungal genera, while ITS2 is reliable and frequently used, it would be best to combine several markers, for a more complete species assignment (Wilson et al., 2023). (iii) Existing errors (mislabeling and sequence quality) in reference databases (Abarenkov et al., 2018). For example, the species name related to a sequence in the database is wrong. This may cause false negatives (the sequence of a threatened species is falsely labeled as a not-threatened one) which would result in a conservative estimation of species numbers by metabarcoding. False positives (the sequence of a not threatened species is falsely labeled as a threatened species), however, would lead to an overestimation of threatened fungal species numbers by metabarcoding. Such misannotated reference sequences are difficult to detect in sampling data, yet the use of curated databases, like UNITE 9.0 (Nilsson et al., 2019) reduces the probability of such mislabels as much as possible.

An alternative explanation of the better performance of metabarcoding relative to fruit body surveys might be that fruit body species identification is false negative. As outlined in the introduction, the identification of fungal species based on their macro-and micromorphological characteristics can be complicated and dependent on available literature. However, fruit bodies were identified by experienced mycologists, particularly well aware of threatened species. That is, as the

macromorphology of a fruit body suggests that this might be a rare species, it would have been sampled for later deeper exploration in the lab using the relevant identification literature. We, therefore, safely assume that false negative identifications based on fruit bodies were less probable.

Taken together, we safely assume that the proportion of detection probability based on metabarcoding is still larger than based on fruit body surveys even if accounting for all mentioned uncertainties. This is further supported by sensitivity analyses using different thresholds of sequence similarity or coverage, as well as randomized or targeted singleton exclusion, yielding robust results (Table B3). Nevertheless, databases should be further developed, first, in species coverage and second, in quality control (Lücking et al., 2020). Thus, comprehensive fungal conservation requires accurate species detection and identification and also the incorporation of fruit body surveys, as long as reference databases are not complete to secure reliable species monitoring and evaluation of the threat status for Red Lists.

4.2 Low number of shared species between study methods

We found that the number of species per site detected by both methods was low (max: ~25%, mean: ~8%) and each method detected a unique set of species. Further, both methods rarely detected species simultaneously on the same object. Besides the already discussed uncertainties of species identification (see 4.1), species traits might explain the detection probability in relation to a specific sampling method: (i) Decrease in detection probability for species or individuals with rather small mycelial sizes (Ovaskainen et al., 2013). Likewise, species with small and/or inconspicuous fruit bodies might be overlooked during fruit body surveys. For example, many corticioid species produce inconspicuous thin layered fruit bodies on the log surface, some preferentially at the bottom of the log which would be visible only if the log would be turned around. Of the 28 corticioid species, 16 were detected via metabarcoding, 7 species by both methods and 5 uniquely by fruit body surveys, supporting this view. (ii) Fruit body surveys might have missed threatened fungi that produce annual short-lived and soft-fleshed fruiting bodies (e.g., Agarics) that depend on a small temporal fruiting window, or exhibit species-specific fruiting phenology (Halme and Kotiaho, 2012; Ovaskainen et al.,

2013; Abrego et al., 2016; but see 4.1 Efficiency of metabarcoding in detecting threatened species). (iii) Soil-related fungi like ectomycorrhizal species (e.g., *Russula)* can inhabit deadwood (Mäkipää et al., 2017; Rajala et al., 2015; Tedersoo et al., 2003), but their ability to produce fruit bodies in this substrate is limited (Holec et al., 2020). Thus, in deadwood, they are more likely to be detected via metabarcoding (Rajala et al., 2011). In general, we detected more non-saprotrophic fungi with metabarcoding (Fig. A2) supporting this view. Further, our results indicate method-specific detectability patterns differing between the species (Table A2-A6). More studies are needed to illuminate the relationship between species or individuals' traits and the detection probability of the respective methods (e.g., Blaschke et al., 2023), to develop more nuanced monitoring advice or target species-specific sampling protocols.

4.3 The impact of decay and host tree identity

Across all objects (in sum), metabarcoding detected more species than fruit body surveys, independent of the deadwood characteristics, decay stage, and tree identity. However, this holds not true on object level, where metabarcoding of low sampling intensity did not outperform fruit body surveys in intermediate decay stages (decay stages two and three). This might be explained by observations that the number of species based on fruit body surveys peaks at intermediate successional stages (Heilmann-Clausen and Christensen, 2003). Thus, in decay stages two and three fruit body surveys perform quite well relative to metabarcoding if sampled at lower levels of intensity. However, although these results suggest that metabarcoding of high sampling intensity produces more reliable sampling data and should be favored if the information at object level is needed, we refrain from overinterpreting these results as a pure sampling intensity effect on object level, due to the confounding effects of sites and metabarcoding sampling intensity.

4.4 Detection probability of objects occupied by threatened species

Metabarcoding detected threatened species on more objects than fruit body surveys. Hence, if a limited set of objects or a small area is sampled for conservation evaluation, metabarcoding should be preferred. One further advantage would be that no expertise in morphological identification would be

needed in the field. However, drilling many large objects with higher sampling intensity in the field and further processing data in the lab can be time-consuming. Additionally, lab expertise and equipment are necessary or expensive if transferred to a sequencing company. On the other hand, if only overall information about the species numbers per site is needed, composite samples (sawdust from several trunks pooled) may reduce such costs, but the risk is to lose some patchily occurring rare taxa (Tedersoo et al., 2022). Alternatively, many more objects can be sampled via fruit body surveys in a shorter time, which may compensate for the lower performance of fruit body surveys at a single object level (Runnel et al., 2015). This can be efficient, in particular, if the aim is to survey threatened species in a large area or on a large number of objects and if the fungal expertise is present. One way of keeping this type of evaluation cheap might be implementing citizen science approaches as many non-professional mycologists can identify fungal species in the field or via microscopy (Heilmann-Clausen et al., 2016). In summary, if the aim is to evaluate the conservation value of a specific site, the method to choose depends on the resources available (e.g., lab capacities, fungal expertise). However, given that each method detected a unique set of species and that both methods rarely detected the same species on the same object, a combined approach might be the most appropriate option.

4.5 Implications for fungal conservation

In this study, we focused on fungal species determined as threatened in Central European Red Lists. Fungal red-listing is rather recent (Mueller et al., 2022) and so far, mainly based on fruit bodies. Our results clearly show that it is important to (i) conduct specific eDNA surveys to improve understanding of the occupancy and distribution of fungi; and (ii) incorporate the increasing body of molecular evidence in fungal red-listing (Větrovský et al., 2020; Tedersoo et al., 2021), especially of those species with no or inconspicuous sexual characteristics. For example, metabarcoding allowed us to detect *Asterostroma cervicolor* in Žofín, a species listed as "extinct or lost" in the Red List of the Czech Republic (Holec and Antonín, 2006). Other species listed in the German Red List as "highly threatened": *Cyphella digitalis*, *Cystostereum murrayi*, *Entoloma hirtipes*, *Hydropus marginellus*, *Phellopilus nigrolimitatus*, *Russula emetica*, *Russula helodes*, and *Russula medullata*, or "threatened

458 with extinction": *Entoloma sericeum* (Dämmrich et al., 2016), were also only detect via
459 metabarcoding in the German sites. Besides all uncertainties in assigning species names correctly via
460 metabarcoding (see above), these observations suggest that some species might be less rare than
461 assumed or have probably a larger distribution than previously assessed (Geml et al., 2014). However,
462 species detection via metabarcoding does not indicate how viable fungal populations are. The present
463 mycelium might only be haploid or only present as spores and thus it remains unclear, whether a
464 fungal individual is established, fit enough for survival, reproduction, and dispersal – or whether it
465 ever will be. Still, this knowledge is important for conservation and threat status assessments. From
466 fruit body surveys, we can be sure that the species is established, and fit enough to reproduce and
467 disperse which is preconditioned to maintain viable populations. Further, macro- and
468 micromorphological characteristics are well-documented, and hence, the probability of a false positive
469 determination seems less pronounced (see above). (iii) Although, overall, metabarcoding
470 outperformed fruit body surveys, we found variability in the relative performance among our analyses
471 (Table B6), most probably attributable to natural differences in site-specific conditions or climatic
472 variability among the years. We, therefore, recommend, independent from the method used to sample
473 threatened species across several years and object characteristics (e.g., decay stages) in a given locality
474 (see also Abrego et al., 2016). (iv) Finally, it is important to emphasize that metabarcoding alone will
475 not resolve all problems of fungal conservation. First, exact results might differ, depending on the
476 chosen sampling and particularly lab procedures. Second, species identification via metabarcoding can
477 be error prone as outlined above. Hence, for species assessment strict quality tests should be followed,
478 and specific training for the handling of metabarcoding data provided. Third, although metabarcoding
479 was overall and per object more efficient than fruit body surveys, it did not detect all species. This
480 seems methodologically inherent and will not resolve with better reference sequences or even higher
481 sampling intensity given that the processing of an entire deadwood object is currently not feasible.
482 Further, fruit body surveys might be more efficient for a rapid survey across many logs in a given area,

depending on the sampling site and available resources (see 4.4). Hence, we might always depend on considering fruit body surveys in addition for comprehensive assessments.

5 Conclusion

We showed that metabarcoding is an efficient technique to detect threatened deadwood-inhabiting fungal species in Central European forests. The efficiency of this technique will further improve with the completion of reference databases and the elimination of errors during the metabarcoding process. Moreover, we demonstrated that the good performance of metabarcoding in species detection bears the potential to gain deeper insights into the distribution of species so far only assessed based on fruit bodies. Hence, this technique seems a valuable tool for further developing different fungal conservation assessments (e.g., Red Lists). However, as some fungal species (incl. those missing sequences in reference databases) were only discovered as fruit bodies, and both methods rarely detected the same species on the same object; we recommend combining both methods for comprehensive assessments of the diversity and distribution of threatened species.

Data availability

This work is partly based on data elaborated by the BELongDead Experiment of the Biodiversity Exploratories program (DFG Priority Program 1374). Data from the sites Alb, Hainich and Schorfheide used for this analysis is publicly available in the Biodiversity Exploratories Information System (https://www.bexis.uni-jena.de/ddm/PublicSearch; accession number: 31703). Data from the Bavarian Forest and Žofín used for this analysis is publicly available at 10.5281/zenodo.12188259. Raw molecular data is available at https://www.ncbi.nlm.nih.gov/sra. Accession numbers: PRJNA756463 (Alb, Hainich and Schorfheide); PRJNA1131312 (Žofín). Data on extracted sequences and metadata for the Bavarian Forest is available at 10.5281/zenodo.12570390.

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748 Table 1: General information about study sites, deadwood objects, sampling methods, and

749 environmental information.

Study site	Established	Number of deadwood objects	Tree species	Object metrics	Sampling years	Metabarcoding sampling intensity and processing reference	Topography/ Climate (mean annual temp./precipitation) Dominant tree species
Alb	2009	117	13	length 4.0 ± 0.25 m (SD) mean diameter 31 ± 5.9 cm (SD)	2012, 2015 2017/18	low one drilling core (Leonhardt et al., 2019)	460-860 m a.s.l. 6-7°C 700-1000 mm *Fagus sylvatica/Picea abies*
Hainich	2009	156	13	length 4.0 ± 0.25 m (SD) mean diameter 31 ± 5.9 cm (SD)	2012, 2015 2017/18	low one drilling core (Leonhardt et al., 2019)	285–550 m a.s.l. 6.5-8°C 500-800 mm *Fagus sylvatica*
Schorfheide	2009	117	13	length 4.0 ± 0.25 m (SD) mean diameter 33 ± 6.5 cm (SD)	2012, 2015 2017/18	low one drilling core (Leonhardt et al., 2019)	3–140 m a.s.l. 8-8.5°C 500-600 mm *Pinus sylvestris/Fagus sylvatica*
Bavarian Forest	2011	67	2	length 5.0 m mean diameter 33 ± 6.5 cm (SD)	2012, 2013, 2015	high four pooled drilling cores, (Baldrian et al., 2016)	650-1450 m a.s.l. 3.5-7.0°C 1300-1900 mm *Fagus sylvatica/Picea abies*
Žofín	No experiment, natural objects used	112	3	Length varying Diameter 30-100 cm	2017	high four drilling cores, two cores pooled, milled, and DNA extracted, subsequently DNA pooled to represent the object (Baldrian et al., 2016)	735-830 m a.s.l. 6.2°C 866 mm *Fagus sylvatica/Picea abies/Abies alba*

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Table 2: Z-values, marginal (R^2m) and conditional (R^2c) R-squares of the generalized linear mixed-effects models, testing the number of threatened species per deadwood object and number of deadwood objects occupied by threatened species detected by fruit body surveys and metabarcoding of low and high sampling intensity and controlling for the deadwood volume as a covariate. Fruit body surveys are the reference group. Asterisks display the significance of each predictor (* = p <0.05, ** = p <0.01, *** = p <0.001).

	Predictors	z value	R^2m/R^2c
Number of species ~	Intercept	-10.02***	0.39/0.39
	Metabarcoding (low)	8.06***	
	Metabarcoding (high)	20.78***	
	Volume	-0.17	
Number of occupied objects ~	Intercept	-4.93***	0.47/0.47
	Metabarcoding (low)	13.06***	
	Metabarcoding (high)	9.83***	
	Volume	4.99***	

759 Table 3: Z-values, marginal (R^2m) and conditional (R^2c) R-squares of the linear mixed-effects models
760 on the number of threatened species detected by fruit body surveys and metabarcoding of low and high
761 sampling intensity in each decay stage and objects of angio-/gymnosperm trees, while controlling for
762 the deadwood volume as a covariate. Fruit body surveys are the reference group. Asterisks display the
763 significance of each predictor (* =p <0.05, ** = p <0.01, *** = p <0.001).

Model group	Number of species ~	z value	R^2m/R^2c
Decay stage 1	Intercept	-10.26***	0.56/0.56
	Metabarcoding (low)	9.05***	
	Metabarcoding (high)	19.77***	
	Volume	-1.16	
Decay stage 2	Intercept	0.52	0.17/0.17
	Metabarcoding (low)	0.45	
	Metabarcoding (high)	3.90***	
	Volume	0.52	
Decay stage 3	Intercept	1.01	0.08/0.15
	Metabarcoding (low)	0.40	
	Metabarcoding (high)	2.71**	
	Volume	0.47	
Decay stage 4	Intercept	-9.56***	0.46/0.46
	Metabarcoding (low)	- - -	
	Metabarcoding (high)	19.11***	
	Volume	1.12	
Angiosperm	Intercept	-6.85***	0.38/0.38
	Metabarcoding (low)	5.41***	
	Metabarcoding (high)	15.00***	
	Volume	-1.30	
Gymnosperm	Intercept	-7.47***	0.42/0.42
	Metabarcoding (low)	6.18***	
	Metabarcoding (high)	14.71***	
	Volume	1.28	

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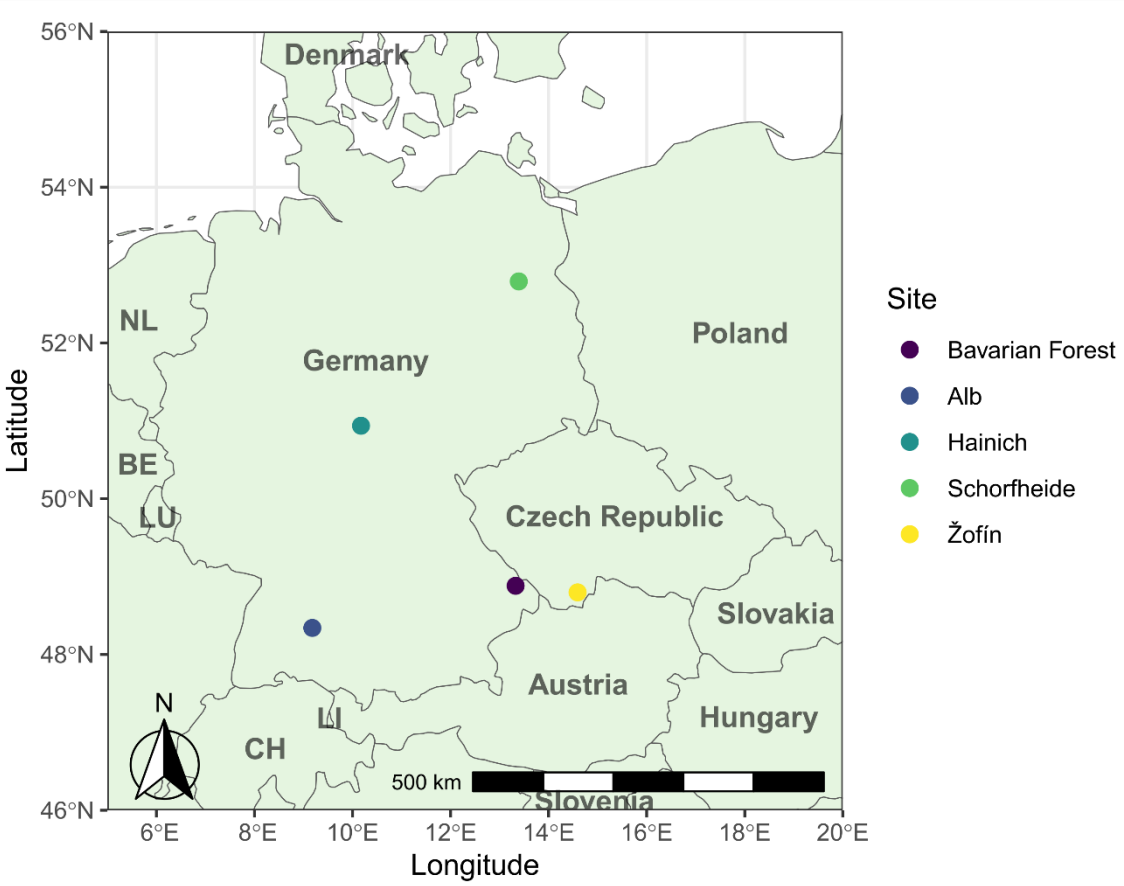

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766 Figure 1: Location of the five sampling sites in Central Europe.

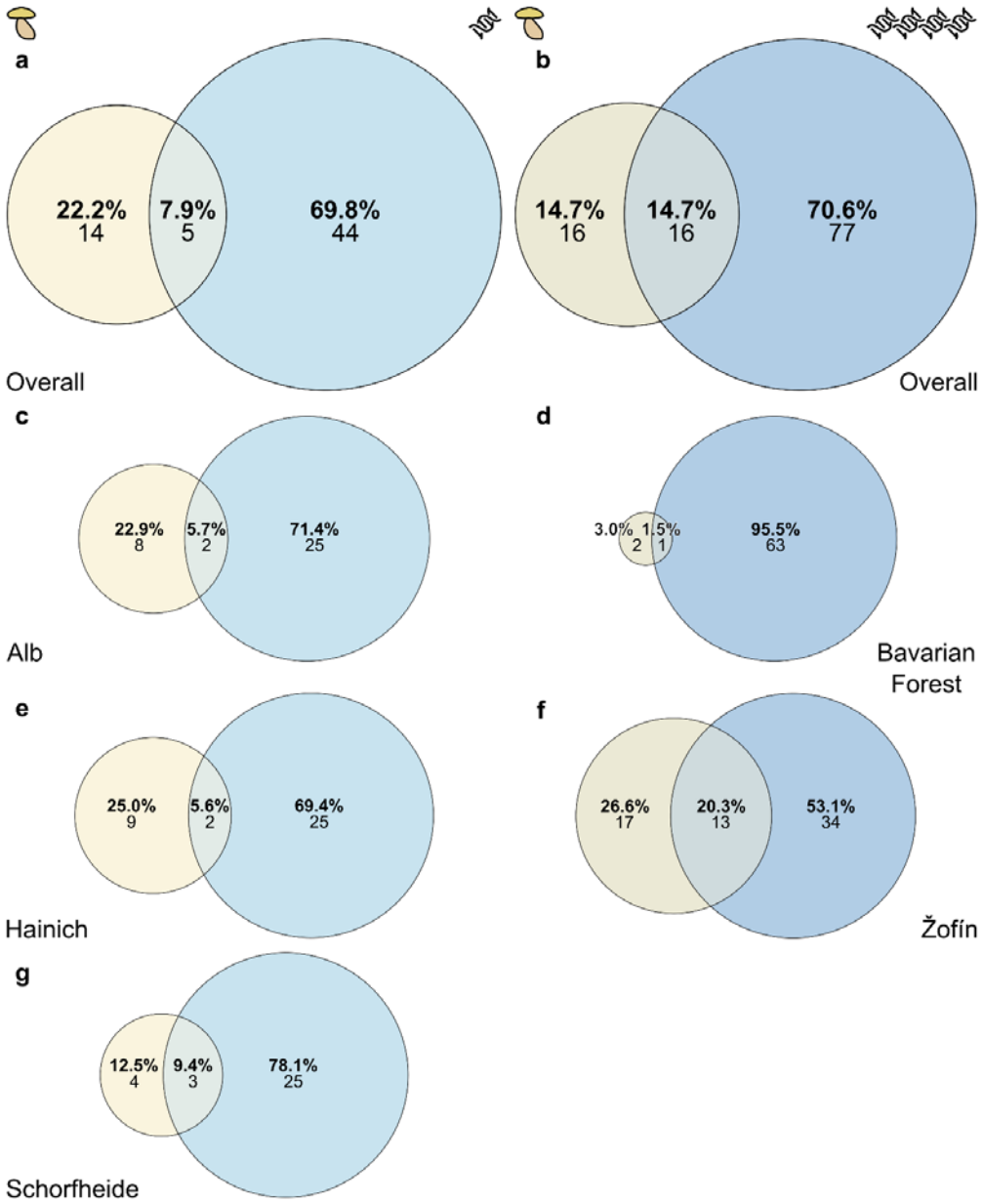
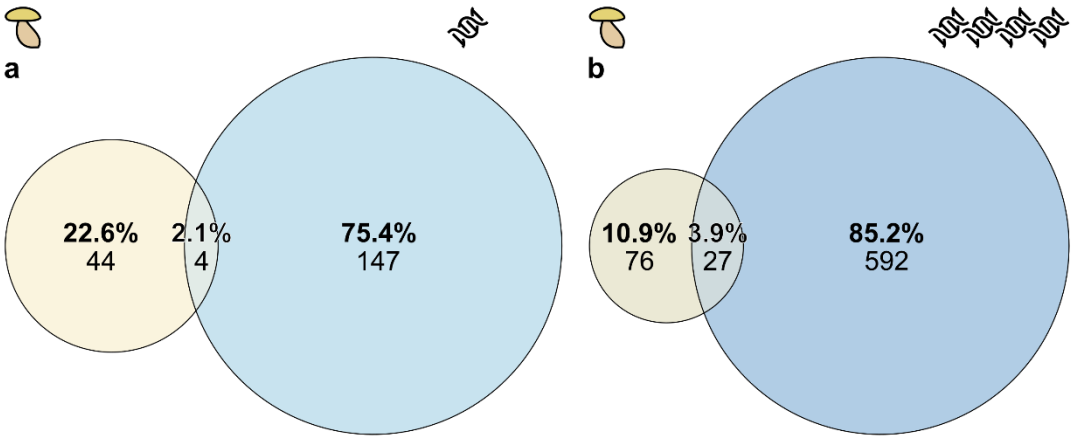


Figure 2: Comparison of the relative and absolute numbers of threatened species detected uniquely by fruit body surveys or metabarcoding with low (a, c, e, g) or high sampling intensity (b, d, f) or by both methods. (a-b) Display the overall comparison for the respective sampling intensity. (c–g) Display the site-specific comparisons.



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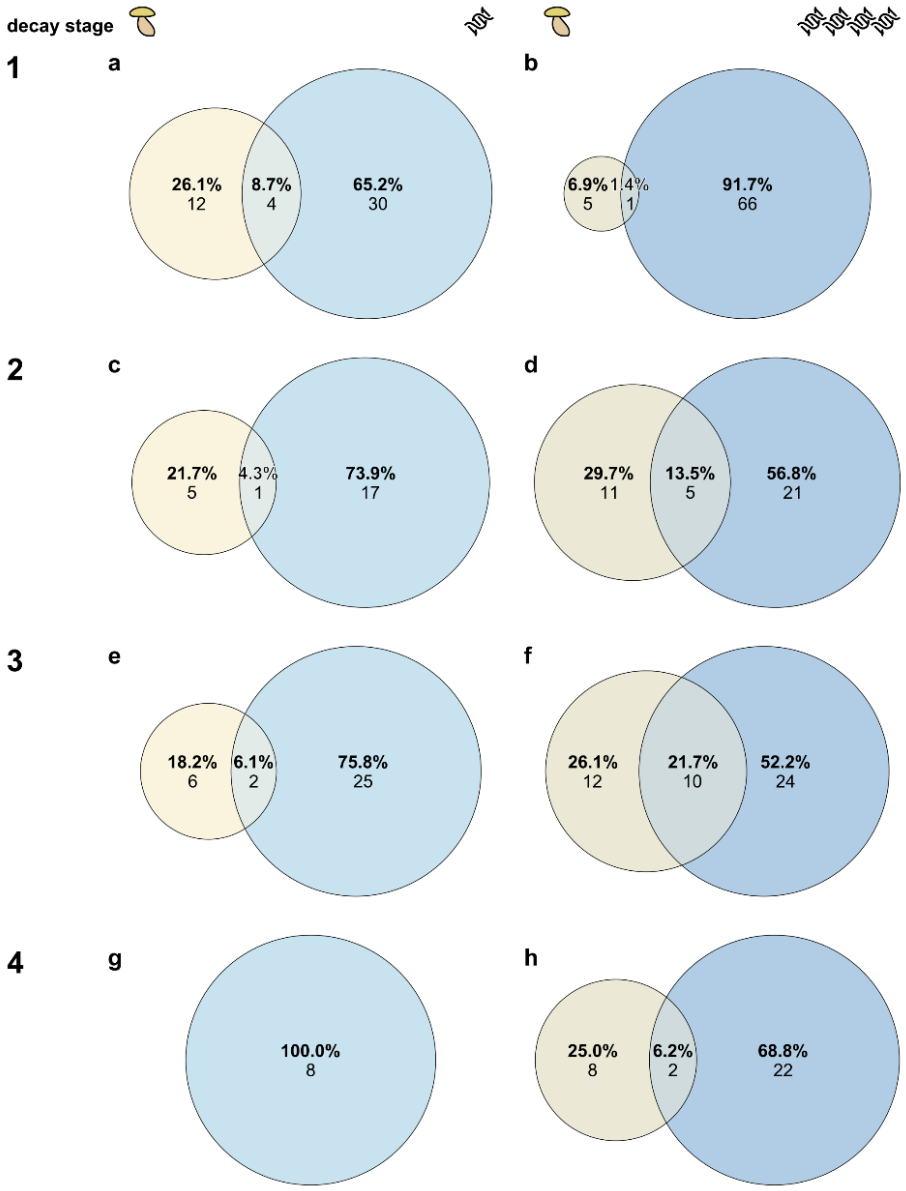
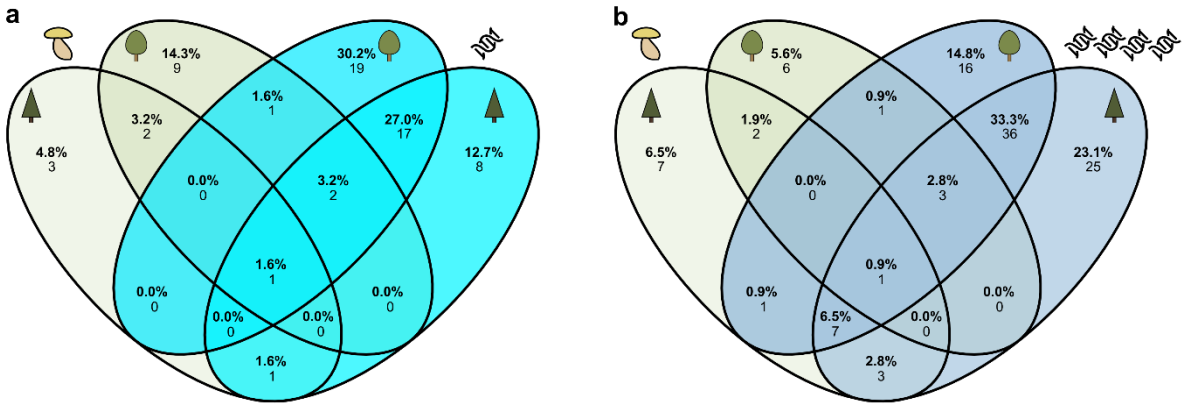
773 Figure 3: Overall comparison of the relative and absolute numbers of deadwood objects on which
774 threatened fungal species were detected across sites, uniquely with fruit body surveys or
775 metabarcoding with low (a) or high sampling intensity (b), or simultaneous by both methods.



776 Figure 4: Overall comparison of the relative and absolute number of threatened species across sites,
777 detected uniquely by fruit body surveys or metabarcoding with low (a, c, e, g) or high sampling
778 intensity (b, d, f, h) or both methods across decay stages.



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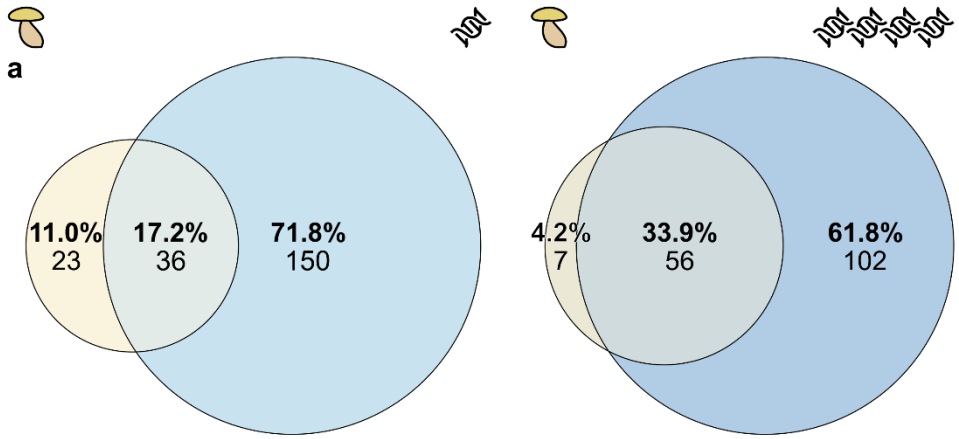
780 Figure 5: Overall comparison of the relative and absolute numbers of threatened species detected on
781 deadwood objects of angio- and gymnosperm tree species across sites, uniquely by fruit body surveys
782 or metabarcoding (a) with low and (b) high sampling intensity, or by both methods.



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784 Figure 6: Overall comparison of the relative and absolute numbers of deadwood objects occupied by
785 threatened species across sites, detected uniquely by fruit body surveys, metabarcoding (a) of low and
786 (b) high sampling intensity, or by both methods.