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Lost in dead wood? Environmental DNA sequencing from dead wood shows little signs of saproxylic beetles

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Abstract

eDNA metabarcoding has become a standard method for assessing wood-inhabiting fungi and bacteria, yet determination of dead-wood-inhabiting beetles still relies on time-consuming collection of beetle specimens. We thus tested whether beetle species can be identified by eDNA sequencing of wood in a mesocosm experiment that manipulated species assemblages. Dead wood samples were taken at exit holes of beetles and DNA was extracted and analyzed using two comparative methods: (i) metabarcoding with standard arthropod primers (421 bp) and (ii) using short species-specific primers (120–264 bp) with Sanger sequencing. Results showed that beetle DNA was amplified by each of the two approaches, however, with (i) we detected only one non-target saproxylic beetle species. In addition, we identified 80 different OTUs with four non-targeted species of arthropods. For (ii) we detected the targeted species in two fresh beetle exit holes out of 20 samples. We suggest that, in contrast to fungi and bacteria, this eDNA metabarcoding approach is not able to reliably detect saproxylic beetles from wood samples, likely due to rapid degradation of their target DNA. Adapting such an approach for large-scale analyses thus requires a better knowledge of degradation processes affecting DNA quality and quantity in wood.

KEYWORDS
dead wood, eDNA metabarcoding, monitoring, *Monochamus sutor*, saproxylic beetles

1 | INTRODUCTION

Metabarcoding of environmental DNA (eDNA) has advanced many opportunities to assess biodiversity. Surveys are based on the genetic identification of taxa and rely on the fact that animals leave DNA traces in their habitat (Taberlet et al., 2012). By sampling the substrate, the targeted taxa can be identified without harming the organisms, and often by minimizing habitat disturbances, which is especially desirable when addressing conservation questions (Barnes & Turner, 2016). Moreover, metabarcoding can support, complement, or even replace traditional sampling methods (Deiner et al., 2017), and is particularly useful for cryptic taxa or where sampling approaches exceed available resources (Biggs et al., 2015). However, although eDNA metabarcoding has been commonly applied in aquatic systems by sampling water, its application in terrestrial ecosystems is only evolving recently (but see Cristescu & Hebert, 2018).

In forest ecosystems, dead wood harbors a large number of species, especially fungi, bacteria, and insects (Stokland et al., 2012).

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eDNA has become a standard approach to detect saproxylic fungi and bacteria (Baldrian et al., 2016; Müller et al., 2020), yet sampling of saproxylic insects still relies on very time-consuming trapping and identification of adult beetles. Traditionally, flight interception, emergence traps, or hand collection (e.g., leaf litter sifting) are used. To obtain representative samples, traps need to be operated over longer time periods and often several sampling methods need to be combined. Analogous to the sampling of fungi and bacteria, metabarcoding of eDNA extracted from dead wood samples has been proposed as a promising approach to sample beetle communities in dead wood over a short time period.

Saproxylic beetles are important agents of wood decomposition (Stokland et al., 2012; Ulyshen, 2016). They are sensitive to forest management and many species have become threatened by forestry practices (Grove, 2002; Paillet et al., 2010; Seibold et al., 2015). In Germany there are around 1500 known species of saproxylic beetles (Möller, 2009), characterized as belonging to different ecological guilds, e.g., xylophages, fungi cultivators, or phloeophages. The larvae of many species build tunnel systems known as galleries within wood or under the bark and then depart from the wood as adults via visible exit holes (e.g., Fierke et al., 2005). Their development time ranges from a few months for smaller species to up to several years for larger species (Gallardo & Cárdenas, 2016). Due to their residence in locally distinct galleries as larvae, traces of their presence (e.g., feces or exuviae) can be specifically sampled by drilling at the exit holes.

Here, we evaluated the potential of eDNA from dead wood to detect saproxylic beetle species. We used a mesocosm experiment, in which experimental logs were colonized by manipulated communities of saproxylic species (Figure 1). This allowed us to assess the presence of specific species in an experimental setting. We first tested (i) if DNA from target species can be sequenced by metabarcoding using the primers BF2/BR2, targeting a 421 bp fragment of the mitochondrial DNA cytochrome oxidase I (COI) gene. Because we did not detect any target species with metabarcoding, we assessed whether this is due to degradation of the beetle DNA into fragments smaller than 421 bp, by employing (ii) a set of newly designed species-specific primers. These primers for *Monochamus sutor* target smaller fragments (120–260 bp) from the COI gene and were Sanger sequenced. Target amplicon lengths for degraded eDNA commonly range between 100 and 250 bp (Meusnier et al., 2008), which can still result in successful species identifications (Hajibabaei et al., 2006). With our study design, we also investigated (iii) whether DNA obtained from old versus fresh exit holes would differ in detectability, as older holes represent older galleries that likely have more degraded DNA.

2 | MATERIAL AND METHODS

2.1 | Field experiment

All samples were taken from logs of a mesocosm experiment located in the Bavarian Forest National Park, in south-eastern Germany. In March 2015, freshly felled spruce logs (16–20 cm in diameter and 50 cm in length) from the same forest stand and without signs of insect presence were placed inside mesh cages to exclude colonizing insects. The cages were placed one meter apart on a total area of around 150 m^2. Living beetles from the same study region were collected using pheromone traps for Scolytinae and by hand collection for Cerambycidae and Buprestidae. These beetles were placed inside the cages to colonize the logs. The

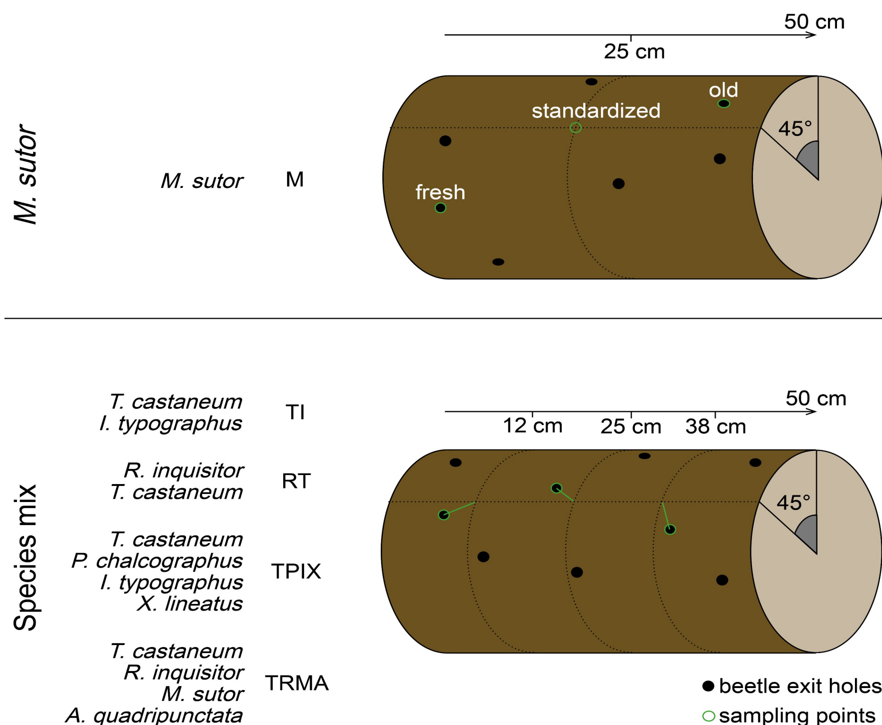

FIGURE 1 Listed are the five species compositions, which were sampled and their respective abbreviations. The sampling procedures for *M. sutor* (top) and the species mixes (bottom) are further outlined on the right

experiment contained five different beetle communities that included either one, two, or four species (Figure 1): M – *Monochamus sutor*; TI – *Tetropium castaneum* and *Ips typographus*; RT – *Rhagium inquisitor* and *T. castaneum*; TPIX – *T. castaneum*, *Pityogenes chalcographus*, *I. typographus*, and *Xyleborus lineatus*; TRMA – *T. castaneum*, *R. inquisitor*, *M. sutor*, and *Anthaxia quadripunctata*. Each beetle community was replicated five times (abbreviated with Ra, Ri, Si, L, Gu) in a randomized block design across the Bavarian Forest National Park, resulting in a total of 24 (5 × 5 minus one missing TPIX log) studied logs. The distance between these replicates was between 2.5 and 11 km.

2.2 | Field sampling

Exit holes were visually inspected and then classified based on their degradation state. Fresh exit holes were characterized by a well-defined outline whose beetles were believed to have emerged in 2017. Old exit holes have started to degrade, i.e., the outlines were already blurry and indistinct. For the latter, beetles were believed to have emerged in 2016.

Sampling was conducted in November 2017 by drilling at the exit holes of beetles and at standardized locations that lacked exit holes to a depth of 3 cm toward the center of the log. Exit holes of *M. sutor* had a mean diameter of 4.0 ± 1.3 mm and their galleries had a mean length of 91.6 ± 68.6 mm as measured by computer tomography scanning (Seibold & Tobisch, 2018). We used an electric drill (Makita) equipped with a 2 × 16 cm wood auger, which was cleaned with ethanol and flamed to avoid any cross-contamination after each sampling. The wood chips from the drilling were collected in clean plastic bags and frozen at −20°C until further processing.

For five logs colonized by *M. sutor*, we took three samples per log in different positions (see Figure 1, top): one at an old (N = 5), one at a fresh (N = 5) exit hole, and one at a standardized location (N = 5; center of the log at a 45° angle to the log axis) that lacked exit holes, resulting in 15 samples for this experiment. For logs colonized by several species (Figure 1, bottom), three samples were taken per log (the hole situated closest to 12, 25, and 38 cm from an end) by drilling at an angle of 45° to the vertical log axis. Subsequently, these three drillings per log were pooled, resulting in 19 samples (four communities with each having five replicates, except for TPIX that was only sampled at four locations).

2.3 | Laboratory methods, sequencing, and bioinformatics

Dead wood was freeze-dried for 8 days at −55°C and homogenized to a fine powder with a swing mill (MM 400, Retsch). Three grams of dead wood powder was used for the DNA extraction with the PowerMax® Soil DNA Isolation Kit (Mo Bio Laboratories), with a few adaptations to the manufacturer's instructions. Library preparation and paired-end sequencing on an Illumina MiSeq (Illumina Inc.) were conducted by Microsynth AG with the standard primers BF2 and BR2 (Elbrecht & Leese, 2017) to amplify a 421 bp long fragment of the mitochondrial cytochrome c oxidase subunit I (COI) region. These primers are known to amplify a broad taxonomic range with a good effective taxonomic resolution for hexapods (Marquina et al., 2019), including all species targeted in this study.

Raw demultiplexed reads were assembled using PEAR 0.9.8 (Zhang et al., 2014), allowing a minimal Phred quality score of 24. We further filtered the assembled FASTQ files with FASTX-Toolkit 0.0.14 (Gordon, 2014) to retain only those reads having a Phred quality score ≥10 for all bases and ≥20 for 95% of all bases. We next excised the primer sites from the sequences with SeqKit (Shen et al., 2016). With USEARCH 7.0.1090 (Edgar, 2010), the assembled and filtered reads were first dereplicated using full-length matching, allowing a minimal cluster size of 1 and then clustered into operational taxonomic units (OTUs) having >97% sequence identity. Each OTU sequence was queried against the NCBI GenBank database using MegaBlast on November 21, 2019, allowing for each sequence up to 8 BLAST matches with an e-value threshold of 0.001. OTUs were discarded if they were <100 bps, if they occurred with <5 reads or if they had an alignment identity <97% to the NCBI GenBank database due to unreliable taxonomic assignments, from now on referred to as sequence identity matches. If the best hit based on percent identity was not assigned to a species (e.g., *Entomobrya* sp. BOLD:ACL6239), we used the next best hit that had a species assignment.

To assess if our quality filtering could have resulted in the loss of Coleoptera specific reads, we repeated our analysis using all of the unfiltered assembled FASTQ reads, and performed primer clipping and assembly with USEARCH. We blasted all unfiltered OTUs against a database that we created from COI reference sequences for European Coleoptera from the Barcode of Life Project (BOLD). Our reference database contained 65,260 unique reference sequences, including all target species. We subsequently filtered the BLAST results for matches that had >97% sequence identity, >5 reads, and >100 aligned bps.

2.4 | Designing and testing of species-specific primers

Species-specific primers that amplified a short region of the mitochondrial COI (120–263 bp) were designed with Geneious Prime 2020.0.4 (Kearse et al., 2012) and tested with touchdown PCR in order to detect degraded DNA fragments of *M. sutor*. PCR was performed with the same DNA extractions that were used in the metabarcoding study for samples that were colonized by *M. sutor* (Table S4). For a detailed description of the primer design and PCR, see Appendix S1 and Table S1. PCR success was assessed on 1.2% agarose gels and Sanger sequenced by Microsynth AG (Balgach, Switzerland).

3 | RESULTS

3.1 | Metabarcoding

Metabarcoding resulted in a total of 611,318 raw reads, which clustered in a total of 22,389 OTUs. Each sample was represented by a mean of 17,980 ± 7696 reads and 659 ± 331 OTUs. After the first filtering step, i.e., OTUs with <5 reads, 19,901 OTUs were discarded. Removing OTUs with <100 bps aligned to the GenBank reference library reduced the dataset to 2410 OTUs and applying the 97% sequence match threshold condensed the final dataset to 80 OTUs (see Table S5). These 80 filtered OTUs belong to three superkingdoms and each sample had a mean of 2.8 ± 2.4 OTUs: Archaea (*N* = 1, unfiltered *N* = 65), Bacteria (*N* = 26, unfiltered *N* = 13,496), and Eukaryota (*N* = 53, unfiltered *N* = 8788). OTUs were assigned to the phylum Ascomycota with 30 OTUs being most common Eukaryota (see Table S6), followed by OTUs without a taxonomic phylum (*N* = 9), and including OTUs belonging to the order Physariida (*N* = 8) and one OTU belonging to Longamoebia, Basiomycota (*N* = 6), and Arthropoda (*N* = 4; see Table 1), Chlorophyta (*N* = 3), and Annelida (*N* = 1) belonging to the species *Chamaedrilus chlorophilus*. Surprisingly, metabarcoding only revealed the presence of one saproxylic beetle, *Crypturgus hispidulus*, which had not intentionally been added to the cages. Other non-target beetle species may have been potentially present as indicated by the unfiltered dataset, which focused on beetles alone (see Table S7). However, these assignments mostly were from a single read and had BLAST match sequence identities of <80% and thus were not reliable.

BLAST of the unfiltered OTUs (22,389) against our reference library of Coleoptera from BOLD resulted in 7446 initial hits. After filtering entries for which <100 bps aligned, 625 hits remained and the filter sequence identity >97% reduced the dataset to three entries. These entries included *C. hispidulus* in three samples but two of them only had one read (sample RT [Ra] and TRMA [Ri]). The presence of *C. hispidulus* in the sample TRMA (Ra) was confirmed via the reference library from BOLD. We further manually blasted the few hits that had between 90% and 97% BLAST sequence identity against the NCBI nucleotide collection and confirmed that these OTUs were not Coleoptera (results not shown).

3.2 | Sanger sequencing

The four primer combinations were applied to the samples (see Table S2), whose logs had been colonized by *M. sutor* (samples M and TRMA). Out of these 20 samples (15 samples containing *M. sutor* alone and 5 with mixed species compositions), two were successfully amplified whose sequences were identified as *M. sutor*, with one primer pair amplifying a 212 bp fragment (see Table S3). The two successfully sequenced samples were two replicates (*N* = 5) of fresh exit holes colonized by *M. sutor* alone.

4 | DISCUSSION

In this study, we tested whether saproxylic beetles can be detected using standard eDNA metabarcoding and Sanger sequencing from dead wood samples making use of a mesocosm experiment containing known species assemblages. Relying on commonly used metabarcoding primers for arthropods, the detection success for beetle species was very low. Only one beetle was detected in the mixed-species samples, while none was detected in the single-species samples with *M. sutor*. When using newly designed species-specific primers for *M. sutor*, which amplify shorter fragments, we were able to detect the target species in two samples from fresh exit holes, but just with one of the four primer pairs. This result suggests that the absence of long DNA fragments, likely due to degradation, is the main reason for not detecting the targeted beetles using our metabarcoding approach, as detection only occurred with the shorter marker.

The selection of primers is crucial in metabarcoding studies (e.g., Elbrecht et al., 2019; Elbrecht & Leese, 2017; Marshall & Stepien, 2019). Here, we used BF2/BR2 that were tested in different studies analyzing bulk samples of a wide range of arthropods (Elbrecht et al., 2019; Marquina et al., 2019) as well as in eDNA studies. Although

TABLE 1 Summary of all arthropod species detected by metabarcoding

	Expected	Observed		
Sample	Species	Class	Family	Species (GenBank ID)
M old (Si)	*M. sutor*	Collembola	Entomobryidae	*Entomobrya nivalis* (HQ943173.1)
		Collembola	Entomobryidae	*Entomobrya corticalis* (LK024455.1)
TRMA (Ra)	*T. castaneum*, *R. inquisitor*, *M. sutor*, *A. quadripunctata*	Insecta	Curculionidae	*Crypturgus hispidulus* (KU918582.1)
TI (Si)	*T. castaneum*, *I. typographus*	Chilopoda	Lithobiidae	*Lithobius borealis* (KX458756.1)

Note: The sample indicates the abbreviation for the species, the characterization of the exit hole (where applicable), and in bracket the geographic replicates.

the primers BF2/BR2 are designed for arthropods, they also amplify some fungi (36 OTUs), as shown in other studies (Macher et al., 2018).

None of the target beetle species were detected with metabarcoding in any of the samples when amplifying the 421 bp fragment. However, we did find *C. hispidulus* in one sample (verified through galleries visualized with computer tomography scans of logs, results not presented here). This species colonized the log accidentally, either because its very small body size (<1.4 mm) allowed it to enter through the mesh or while opening cages for measurements. In both cases, it is likely that *C. hispidulus* colonized the log later than the target beetles did. It might thus still have been active at the time of sampling or emerged shortly before. The other detected arthropod species (Collembola and Chilopoda) can be found in many different habitats, but typically are found in the litter layer (Ferro, 2018). They might have entered the mesocosms the same way as did *Chamaedrilus chlorophilus*, the detected annelid species. Annelids are known to inhabit wood and contribute to degradation (Dózsa-Farkas, 2019; Zuo et al., 2021).

Our result suggests that the rate of DNA degradation in dead wood is high. Similar to soil, degradation likely depends on chemical, physical, and biological properties of the substrate (Levy-Booth et al., 2007). The interaction of humic acids, cation concentration, and pH with DNA can influence the persistence by reducing the risk of degradation through DNase and nuclease. Barnes and Turner (2016) additionally highlight the importance of the biotic environment (i.e., composition and activity of the microbial community and extracellular enzymes). In dead wood, fungi are major agents of wood decomposition with their extracellular enzymes (Kahl et al., 2017) probably influencing the persistence of DNA. Fungal colonization of wood is promoted by saproxylic beetles that carry spores and introduce them to new wood, where beetle entry holes serve as entrance ports for fungi (Jacobsen et al., 2017; Seibold et al., 2019). Fungal activity and thus DNA degradation may therefore be particularly high near beetle galleries. In addition, the pH of dead wood is acidic, ranging between 3.2 and 6.0 (Baldrian et al., 2016), probably accelerating DNA degradation. As in many other eDNA metabarcoding studies the persistence of DNA in time and space is important to infer conclusions, but so far the mechanisms of degradation are not well understood (Barnes & Turner, 2016). However, experiments of DNA degradation in water have shown an exponential decay or even a decay constant that decreases over time.

The detection of *M. sutor* on logs ($N = 20$) using shorter markers was only partly successful. First, the species might not have been sampled in the logs colonized by several species (TMRX, $N = 5$) as the three exit holes sampled might have belonged to other beetle species. Second, we did not expect any *M. sutor* DNA at the random, standardized location, which indeed we did not find. We did however expect to find DNA at the old and fresh exit holes. After all, we were only able to amplify short fragments of the target DNA at fresh exit holes. The sensitivity of high-throughput sequencing, as metabarcoding, is higher compared to Sanger sequencing, implicating that metabarcoding could even detect rare target DNA more reliably (Meyerson et al., 2010). Nevertheless, we suppose that DNA in dead wood is degrading quickly, so only individuals which were recently active at the exact location where the sampling took place can be detected. The spatial restriction and the origin of the DNA is a likely reason why our eDNA metabarcoding of beetles was less successful than for fungi and bacteria, which are widespread in dead wood. In addition only short fragments should be targeted, as they may be more likely to be recovered (Bylemans et al., 2018). Primers specifically designed for beetles and for another gene alternatively may be more successful, and should be tested.

With the applied methods, we showed that there is detectable beetle DNA in dead wood. For now, the presented methods do not allow for assessments of whole communities of beetles, as is possible for fungi and bacteria. While fungi and bacteria are widespread in dead wood, beetle galleries are much more spatially restricted. However, the fact that we detected beetle species when drilling at exit holes is very promising. Adaptation and improvement of sampling procedures, DNA extraction protocols, and lab settings may help to increase the recovery rate in the future. Such a method will only be successful if target DNA can be more effectively sampled and if beetles were recently active in the substrate.

The eDNA metabarcoding approach, if successfully modified, would not only allow species detection and assessment but with the combination of several primers targeting different taxonomic groups would allow the investigation of more detailed hidden interactions in dead wood ecology.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JM and SS designed the experimental setup. JM, NW, SS, and GS developed the idea. NW (with help from assistants) collected and processed data. KL analyzed raw sequences. NW wrote the manuscript with advice from all authors which finally gave approval for publication.

DATA AVAILABILITY STATEMENT

Raw sequence data is deposited on NCBI (Bioproject ID PRJNA798613). Blast output of all samples and OTUs is stored on DRYAD (https://doi.org/10.5061/dryad.37pvmcvmt).

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SUPPORTING INFORMATION

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